UNIVERSITÉ DES ANTILLES ET DE LA GUYANE

HABILITATION À DIRIGER DES RECHERCHES

Soutenue publiquement le : juin 2007

Sujet :

Etude des maladies parasitaires: De la dynamique des populations des agents phytopathogènes aux mécanismes et gènes de résistance de la plante hôte.

Par

Dr. Jean Heinrich DAUGROIS

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Chapitre 1 : Curriculum Vitae

1.1 Etat civil

Nom : **Daugrois**

Prénom : Jean Heinrich
Nationalité : Française
Date de naissance : 26 juillet 1961
Lieu : Paris XV

Situation familiale : Marié, 2 enfants.

Adresse professionnelle : Cirad, UPR75
Station de Roujol
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Profession : Phytopathologiste (Dr en Biologie et Technologie végétale)

Fonction : Responsable service pathologie canne à sucre Guadeloupe,
Responsable de site, Station de Roujol, Cirad, Petit Bourg,
Centre de Coopération Internationale en Recherche Agronomique pour le Développement.

Compétences : Diagnostic, identification des maladies, épidémiologie et variabilité des agents pathogènes chez *Saccharum spp*. et évaluation de la résistance aux maladies.

Régions d'expérience : Caraïbes, Océan indien

Diplôme : Doctorat d'Université,
Spécialité : Biologie et technologie végétales. Option phytopathologie.
Université Paul Sabatier / Toulouse / France (1989)

DEA de Biologie et Technologie Végétale.
Université Paul Sabatier/Toulouse/France (1986)

Compétences linguistiques : Anglais, Allemand

1.2 Expériences professionnelles :

- **Thèse de Doctorat de l'Université** (spécialité biologie et technologie végétales, option phytopathologie) préparée au Centre de Physiologie Végétale, U.R.A. C.N.R.S. 241 à l'Université Paul Sabatier, à Toulouse, sous la direction du Professeur A. Thouzé. (Août 1987 – Décembre 1989)

  Sujet : Les β-1,3-glucanases du haricot, leur implication dans la résistance à l'anthracnose.

- **Phytopathologiste au Cirad Irat Réunion** (Avril 1990 – Juin 1992) **puis au Cirad-ca Réunion** (Juillet 1992 – Juin 1993).
  Responsable d'une équipe comprenant 1 technicien et 2 collaborateurs.

  Sujet : les maladies de la canne à sucre
  a) Contrôle sanitaire
  b) Sélection de variétés résistantes, mise au point de tests d'évaluation.
  c) Etude génétique de la résistance à la rouille.

- **Phytopathologiste au Cirad-ca Guadeloupe** (Août 1993 – Juin 2000)
  Responsable d'une équipe comprenant 2 techniciens, 1 VAT (1995 – 1997) et du laboratoire de pathologie canne à sucre hébergé au sein du laboratoire de biologie cellulaire et culture in vitro du Cirad-ca, station de Roujol, Petit Bourg.

  Sujet : les maladies de la canne à sucre
  a) Contrôle sanitaire, inventaire des maladies, impact sur la production.
  b) Sélection de variétés résistantes.
  c) Etude de l'échaudure des feuilles provoquée par *Xanthomonas albilineans* (variabilité de l'agent pathogène, épidémiologie, méthodes de lutte).

- **'Visiting scientist' au département d'agronomie et environnement, Centre Agricole Université de Louisiane, Baton Rouge**, LA 70803, USA (Août 2000 – Juin 2001)

  Sujet : Effet d'une nouvelle famille d'herbicide sur la maladie de pourriture de racine de la canne à sucre provoquée par *Pythium* sp.

- **Phytopathologiste au Cirad-ca Guadeloupe** (à compter de Septembre 2001)
  Responsable d'une équipe comprenant 2 techniciens, 1 VAT (1995 – 1997) et du laboratoire de pathologie canne à sucre hébergé au sein du laboratoire de biologie cellulaire et culture in vitro du Cirad-ca, station de Roujol, Petit Bourg.

  Sujet : Les maladies de la canne à sucre
  a) Contrôle et veille sanitaire
  b) Sélection de variétés résistantes.
  c) Etude de la maladie de la feuille jaune provoquée par le SCYLV ou *Sugarcane Yellow Leaf Virus* (épidémiologie, variabilité de l'agent pathogène, étude du complexe virus – vecteur – plante et résistance variétale, préconisation de méthode de lutte).
  d) Etude de l'échaudure des feuilles provoquée par *Xanthomonas albilineans* (épidémiologie, résistance variétale, variabilité génétique de l'agent pathogène et agressivité).

Collaborations scientifiques :

- UMR BGPI Montpellier –P. Rott et M. Royer (Variabilité des agents pathogènes, *X. albilineans* et SCYLV) ; E. Muller (Etude des Badnavirus). N. Sauvion (Etude du complexe Plante – vecteur –virus, cas du SCYLV)
- UAG, département Mathématique et informatique, J. Vaillant (épidémiologie du SCYLV, dynamique des populations du puceron vecteur et contamination des plantes).
- Université de Louisiane, J. Hoy (Impact de *Pythium* sp. sur la culture de la canne à sucre).
- WICSCBS (West Indies Central Sugarcane Breeding Station), Barbados.

Expertise :

- Belize Sugar Industry, Belize, Février 2006, 6j, Examen sanitaire de l'exploitation du BSI quand à la présente de maladies à caractère systémique.
- SIRI Jamaïque, Mai 1999, 6 j. Examen sanitaire des exploitations sucrières.
- Tchad, Juin.,1998. Expertise phytosanitaire de l'exploitation sucrière de la SONASUT au Tchad.
- Rwanda, Octobre 1991, 8 j.Examen sanitaire des plantations et inventaires des maladies présentes. (Technisucre)
- Madagascar, Mai 1991, 15 j. Constat d'éradication de la maladie de Fiji. (FAO)

1.3 Activités d'encadrement :

1.3.1 Thèse

En co-direction avec P. Rott – CIRAD : M. Patrice Champoiseau, 2003-2006. Ecole Doctorale U. Antilles-Guyane. Diversité génétique et biodiversité de *Xanthomonas albilineans* en Guadeloupe et variabilité de la pathogénie.

En co-direction avec J. Vaillant – UAG : Mme Carine Edon Jock, 2004-2007. Ecole Doctorale U. Antilles-Guyane. Spécificité de la dissémination du virus de la feuille jaune de la canne à sucre aux Antilles françaises.

1.3.2 DEA :

M. Patrice Champoiseau, janvier - août 2002. DEA Ressources Phytogénétiques et interactions Biologiques, U. MontpellierII/ENSAM. Capacité colonisatrice et diversité génétique de *Xanthomonas albilineans*, agent causal de l'échaudure des feuilles.

Mlle Carine Edon, janvier - juin 2004. DEA Environnement tropical et valorisation de la biodiversité, Faculté des Sciences Exactes et Naturelles, U. Antilles-Guyane. Maladie de la feuille jaune de la canne à sucre : analyse de la dissémination du virus et caractérisation de cultivars résistants au vecteur.

M. Frédéric Caray, mars - août 2004. DEA Sciences Agronomiques, INPL Nancy.

Rotation bananier - canne à sucre – bananier : biodiversité et évolution biologique du milieu cultivé.

1.3.3 Ecole d'ingénieur :

M. Damien Hillard, mars – septembre 1996. ENSAIA Nancy. Rabougrissement des repousses de la canne à sucre : Etude de la dynamique bactérienne de l'agent causal *Leifsonia xyli* subsp. *xyli*. (Préparation et vérification des différents outils méthodologiques destinés au criblage variétal.)

Mlle Valérie Dumont, mai – décembre 1997. ESA Purpan. Etude de la survie épiphyte de *Xanthomonas albilineans* sur feuilles de canne à sucre (*Saccharum* spp)

M. Patrice Champoiseau, juin – décembre 1999. ISTOM. Etude de l'épidémiologie de l'échaudure des feuilles de la canne à sucre (*Saccharum* spp) causée par *Xanthomonas albilineans.*

1.3.4 Maîtrise :

Jean-Laurent Baflast, mai – juin 2000. Maîtrise de biologie des populations et écosystèmes, Faculté des Sciences Exactes et Naturelles, U. Antilles-Guyane. La résistance au SCYLV des ressources génétique canne à sucre du CIRAD Guadeloupe.

Carine Edon, mai – juin 2003. Maîtrise de biologie des populations et écosystèmes, Faculté des Sciences Exactes et Naturelles, U. Antilles-Guyane. Etude de la résistance variétale de la canne à sucre au rabougrissement des repousses.

Sylvia Beramis, mai – juin 2004. Maîtrise de biologie des populations et écosystèmes, Faculté des Sciences Exactes et Naturelles, U. Antilles-Guyane. Caractérisation des géniteurs utilisés dans le programme de sélection de variétés de canne à sucre quant à la résistance à deux maladies systémiques causées par le SCYLV et *Leifsonia xyli* subsp. x*yli*.

1.3.5 BTS :
Koba Brancourt, mai – juillet 2000. LEGTA d'Aix-Valabre. Contrôle des maladies systémiques dans les pépinières de canne à sucre.

1.4 Activités d'enseignement :

Université de la Réunion, Maîtrise de Chimie et Biologie Végétale :

- Cours, 1992-1993 :
  - Interaction plantes microorganismes pathogènes (5 h)

Université Antilles-Guyane, MST Sciences agronomiques et développement rural, Faculté des Sciences Exactes et Naturelles :

- Cours :
  - Les bactéries phytopathogènes, 4 heures, année 1999
  - Les maladies de la canne à sucre dans la zone caraïbe (2h / session)
- TD Agronomie :
  - Session 1997-1998 : 6 étudiants, 8 séances de 4 h.
  - Session 1999-2000 : 2 groupes de 4 et 5 étudiants, 10 journées.

Université Antilles-Guyane, Master Mathématiques appliqués, Faculté des Sciences Exactes et Naturelles (depuis 2006):

- **A**pplication des statistiques en biologie (cours, 6 heures)

1.5 Liste des publications :

1.5.1 Publications internationales à comité de lecture :

Articles de revues internationales à facteur d'impact:

1. Abu Ahmad Y., Costet L., **Daugrois J.H.,** Nibouche S., Letourmy P., Girard J.C., and Rott P. 2007. Variation in infection capacity and in virulence exists between genotypes of *Sugarcane yellow leaf virus*. Plant Disease, 91: 253-259.

2. Abu Ahmad Y., Royer M., **Daugrois J.-H.,** Costet L., Lett J.-M., Victoria J. I., Girard J.-C., and Rott P., 2006. Geographical distribution of four *Sugarcane yellow leaf virus* genotypes. Plant Dis. 90:1156-1160.

3. Champoiseau P., **Daugrois J.-H.,** Pieretti I., Cociancich S., Royer M. and Rott P., 2006. High variation in pathogenicity of genetically closely related strains of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen, in Guadeloupe. Phytopathology 96:1081-1091.

4. Falloon T., Henry E., Davis M.J., Fernandez E., Girard J.-C., Rott P., **Daugrois J.-H**., 2006. First report of *Leifsonia xyli* subsp. *xyli*, causal agent of ratoon stunting of sugarcane, in Jamaica. Plant disease vol.90:n°2, p. 245.

5. Champoiseau P., **Daugrois J.-H**., Girard J.-C., Royer M., Rott P., 2006. Variation in albicidin biosynthesis genes and in pathogenicity of *Xanthomonas albilinean*s, the sugarcane leaf scald pathogen. Phytopathology, vol.96:n°1, p. 33-45

6. **Daugrois J.-H.,** Hoy J.W., Griffin J.L., 2005. Protoporphyrinogen oxidase inhibitor herbicide effects on pythium root rot of sugarcane, *Pythium* species, and the soil microbial community. Phytopathology, vol.95:n°3, p. 220-226.

7. **Daugrois** J.H., Dumont V., Champoiseau P., Costet L., Boisne-Noc R., Rott P., 2003. Aerial contamination of sugarcane in Guadeloupe by two strains of *Xanthomonas albilineans*. European journal of plant pathology, vol.109, p. 445-458.

8. **Daugrois** J.H., Champoiseau P., Boisne-Noc R., Rott P., 2000. Variability in pathogenicity of *Xanthomonas albilineans* in Guadeloupe. Phytopathology, vol. 90 n°6, S18

9. **Daugrois** J.H., Jean-Baptiste I., Lockhart B.E.L., Irey S., Chatenet M., Rott P., 1999. First report of *sugarcane yellow leaf virus* in French West Indies. Plant Disease, vol. 83, n. 6, p. 588.

10. Feldmann P., **Daugrois** J.H, Davis M., Chatenet M., Rott P., 1997. First report of leaf scald disease and ratoon stunting disease of sugarcane in French Guyana. Plant Disease, vol. 81, n. 6, p. 696.

11. **Daugrois** J.H., Grivet L., Roques D., Hoarau J.Y., Lombard H., Glaszmann J.C., D'Hont A., 1996. A putative major gene for rust resistance linked with a RFLP marker in sugarcane cultivar R570. Theoretical and Applied Genetics, vol. 92, p. 1059-1064.

12. **Daugrois** J.H., Saed Mohamed B., Rott P., 1995. Screening sugarcane germplasm for resistance to leaf scald disease by analysis of pathogen population densities. Phytopathology, vol. 85, n° 12, p. 1562

13. **Daugrois** J.H., Lafitte C., Barthe J.P., Faucher C., Touzé A., Esquerre Tugaye M.T., 1992. Purification and characterization of two basic Beta-1,3-glucanases induced in *Colletotrichum lindemuthianum* infected bean seedlings . Archives of Biochemistry and Biophysics, vol. 292, n. 2, p. 468-474

14. **Daugrois** J.H., Lafitte C., Barthe J.P., Touzé A., 1990. Induction of Beta-1,3-glucanase and chitinase activity in compatible and incompatible interactions between *Colletotrichum lindemuthianum* and bean cultivars . Journal of Phytopathology, vol. 130, n. 3, p. 225-234

1.5.2 Publications sans comité de lecture :

Article de revue :

1. **Daugrois** J.H., Hoy J.W., Griffin J.L., 2002. Protox Inhibitor Herbicide Effects on *Pythium* and Root Rot of Sugarcane. Journal American Society of Sugarcane Technologists, vol. 22, p 131.

2. **Daugrois** J.H., Bonotto S., Siegwart M., Joseph S., and Rott P, 2004. Spread of sugarcane yellow leaf virus in a healthy sugarcane field and associated populations of *Melanaphis sacchari* in Guadeloupe. Fitopathologia, vol.39:n°2, p98.

Articles de Proceedings et recueils :

15. **Daugrois** J.H., Champoiseau P., Boisne-Noc R., Rott P, 2005. Epiphytic colonization and infection by *Xanthomonas albilineans* of two sugarcane cultivars differing in resistance to leaf scald disease. Proceedings of the XXV congress of International Society of Sugarcane Technologists**,** Guatemala City, Guatemala, 2005. Proc. Int. Soc. Sugar Cane Technol. 25:678-685.

3. **Daugrois** J.H., Jean-Baptiste I., Rott P., 1999. Partial association of sugarcane yellow leaf syndrome symptoms and S*ugarcane yellow leaf virus* in the French West Indies. In Proceedings of the XXIII Congress of International Society of Sugarcane Technologists, New-Delhi, India, 22-26 February 1999, vol. II, 401-403.

4. Rott P., **Daugrois** J.H., Girard J.C., 1996. Major diseases affecting sugarcane production in Guadeloupe and Réunion Island, and recent experience with sugarcane diseases in quarantine at CIRAD-CA in Montpellier. In : Croft B.J., Piggin C., Wallis E.S., Hogarth D.M., Sugarcane germplasm, conservation and exchange. Report of an international workshop. Canberra, Australie, ACIAR, p. 49-50. International Workshop on Sugarcane Germplasm, Conservation and Exchange, 1995/06/28-30, Brisbane, Australie. ACIAR Proceedings.

5. **Daugrois** J.H., Letourmy P., 1996. Quantitative method for studying sugarcane resistance to *Puccinia melanocephala*. In : Proceedings of the XXII congress of International

society of sugarcane technologists, Cartagena, Colombia, 11th-15th September 1995, Vol. 2, p.548-556.

6. D'Hont A., Grivet L., Lu Y.H., Roques D., Feldmann P., Rao P.S., **Daugrois** J.H., Dufour P., Berding N., Walker D.I.T., Hamon P., Glaszmann J.C., 1996. The genome of modern sugarcane varieties. In: Proceedings of the XXII congress of International society of sugarcane technologists, Cartagena, Colombia, 11th-15th September 1995, Vol. 2, p.363-367

1.5.3 Chapitre d'ouvrage :

1. Rott P., **Daugrois** J.H., 2000. Apex rot. In: A Guide to Sugarcane Diseases (Ed. Rott, P, Bailey, RA, Comstock, JC, Croft, BR & Saumtally, AS). CIRAD and ISSCT. Repères – Cirad Montpellier France.

1.5.4 Communications à Congrès :

1. Jacquet O., Edon C., Vaillant J., and **Daugrois J.-H.,** 2006. Statistical study of Spatio-temporal evolution of plant infection by SCYLV in a disease free plot. 15th Annual Meeting of the Caribbean Academy of Sciences, Gosier, Guadeloupe May 21-23, 2006.

2. Champoiseau P., **Daugrois J.-H.,** Royer M., Rott P., 2006. Vers l'identification des gènes impliqués dans la pathogénie de *Xanthomonas albilineans*, l'agent causal de l'échaudure des feuilles de la canne à sucre. In Résumés des Septièmes Rencontres Plantes Bactéries, 20-24 mars 2006, Aussois, INRA Centre de Recherche d'Angers, France, p. 46.

3. Champoiseau P., **Daugrois J.-H.,** Royer M., Rott P., 2006. Towards identification of genes involved in pathogenicity of *Xanthomonas albilineans*, the sugarcane pathogen. VIIIth ISSCT Pathology Workshop Petit-Bourg, Guadeloupe (FWI), 23 - 27 January 2006

4. Carine Edon, Jean Vaillant, Nicolas Sauvion and **J.H. Daugrois**, 2006. Spatiotemporal evolution of plant infection by SCYLV in a disease free plot. Toward modeling virus spread in tropical conditions. VIIIth ISSCT Pathology Workshop Petit-Bourg, Guadeloupe (FWI), 23 - 27 January 2006

5. **Daugrois J.-H.,** Champoiseau P., Rott P., 2006. Impact of rainfall on epiphytic colonization of sugarcane by the leaf scald pathogen and associated plant infection. VIIIth ISSCT Pathology Workshop Petit-Bourg, Guadeloupe (FWI), 23 - 27 January 2006

6. Abu Ahmad Y., Royer M., Costet L., **Daugrois J-H.,** Lett J-M., Victoria J.I. and Rott P, 2006. Genotyping of *Sugarcane yellow leaf virus* in Colombia, Guadeloupe and Reunion VIIIth ISSCT Pathology Workshop Petit-Bourg, Guadeloupe (FWI), 23 - 27 January 2006

7. Champoiseau P., Renier A., **Daugrois J.-H**., Royer M., Rott P., 2005. Identification de marqueurs moléculaires associés à la recrudescence de l'échaudure des feuilles de la canne à sucre causée par *Xanthomonas albilineans*. In Résumés du 6e congrès de la société Française de Phytopathologie, 23-25 février 2005, Toulouse, INRA, CNRS, INP-ENSAT, Université Paul Sabatier, France, p. 41.

8. **Daugrois J.-H**.; Champoiseau P.; Rott, P., 2005. Epiphytic colonisation and infection by Xanthomonas albilineans of two sugarcane cultivars differing in resistance to leaf scald disease. In : International Society of Sugar Cane Technologists Proceedings of the XXV Congress, 30 January - 4 February 2005, Guatemala / Ed. Hogarth. - Guatemala : ATAGUA, 2005. - - p. 678-685. ISSCT Congress

9. Abu Ahmad, Y.; Royer, M.; Costet, L.; **Daugrois, J.-H**.; Lett, J.-M.l; Rott, P., 2005. Genotypage du sugarcane yellow leaf virus présent en Guadeloupe et à la Réunion. In : Société française de phytopathologie, VIème congrés, 23-24-25 février 2005, Toulouse : programme et résumés des communications. - Paris : Société française de phytopathologie, 2005. - [1] p.

10. Champoiseau P., **Daugrois J-H.,** Girard J-C., Royer M., Rott P., 2004. Diversité génétique et variabilité intraspécifique du pouvoir pathogène de *Xanthomonas albilineans*, agent causal de l'échaudure des feuilles de la canne à sucre. In Résumés des Sixièmes Rencontres Plantes-Bactéries, 11-15 janvier 2004, Aussois, UMR GDPP, IBVM, INRA et Université Victor Segalen Bordeaux 2, France, p. 87

11. **Daugrois J.H.,** Roques D. and Rott P., 2004. Evolution of sugar cane leaf scald in Guadeloupe and its impact on disease management. 28th West Indies Sugar Technologists Conference, Barbados, 2004.

12. **Daugrois J.H**., Bonotto S., Siegwart M., Joseph S., and Rott P., 2004. Spread of Sugarcane yellow leaf virus in a healthy sugarcane field and associated populations of *Melanaphis sacchari* in Guadeloupe. 44th Annual meeting of American Phytopathology society, Caribbean Division, 24-28 May, Havana, Cuba.

13. **Daugrois J.H**., Bonotto S., Siegwart M., Joseph S., Rott P., 2003. Infection of sugarcane by SCYLV associated with high populations of *Melanaphis sacchari* in Guadeloupe. ISSCT sugarcane pathology workshop, 7, May 2003, Louisiana State University.

14. **Daugrois J.H**., Boisne-Noc R., Champoiseau P., Dumont V., Rott P, 2003. Epiphytic life of *Xanthomonas albilineans* is involved in the infection process of sugarcane by leaf scald in Guadeloupe. ISSCT sugarcane pathology workshop, 7, May 2003, Louisiana State University.

15. Muller E., Laboureau N., Harper G., Seal S., **Daugrois J.H**., Teycheney P.Y., 2003. Diversité génétique des Badnavirus . In : Pierre Yot ; CNRS, 9ème Rencontres de virologie végétale. Strasbourg, France, CNRS, 2003/02/02-06, Aussois, France.

16. **Daugrois J.H**., Boisne-Noc R., Rott P., 2000.Testing sugarcane for resistance to *sugarcane yellow leaf virus*. The 6th ISSCT Sugarcane Pathology Workshop, 2000/07/17-23, Cha-am, Thaïlande.

17. **Daugrois J.H**., Champoiseau P., Boisne-Noc R., Rott P., 2000. Variability in pathogenicity of *Xanthomonas albilineans* in Guadeloupe. APS annual meeting, August 12-16, 2000, New Orleans, Louisiana.

18. Oriol P., Roques D., **Daugrois J.H.**, Gelabale G., 2000. L'amélioration variétale de la canne à sucre en Guadeloupe : dispositif et perspectives d'évolution. Premier atelier

régional sur l'amélioration variétale de la canne à sucre en Afrique de l'Ouest et Centrale : 1999/10/05-08, Yamoussoukro, Côte d'Ivoire.

19. Roques D., Negroni L., Robin S., Toubi L., Gelabale G., **Daugrois J.H**., Oriol P., Rott P., Feldmann P., 2000. Feasibility of inoculating seedlings with smut at first stage of selection. 6th ISSCT Breeding workshop, 2000/11/13-17, Bridgetown, Barbados.

20. **Daugrois** J.H., Jean-Baptiste I., Rott P., 1999. Partial association of sugarcane yellow leaf syndrome symptoms and *sugarcane yellow leaf virus* in the French West Indies. XXIII ISSCT Congress, New-Delhi, India, 22-26 February 1999.

21. **Daugrois** J., Costet L., Rott P., 1998. Resistance to leaf scald disease in wild relatives of sugarcane analysed by pathogen population densities. International Congress of Plant Pathology. 7, 1998/08/09-16, Edinburgh, Royaume-Uni.

22. D'Hont A., Grivet L., Asnaghi C., Paulet F., Roques D., Jannoo N., Alix K., Piperidis G., Girard J.C., **Daugrois J.-H,** Feldmann P., Glaszmann J.C., 1998. Progress in sugar cane genome analysis. 4th ISSCT Breeding and Germplasm Workshop. 1998/05/18-22, Le Réduit, Mauritius.

23. Feldmann P., **Daugrois J.-H**, Oriol P., Rott P., 1998. Micropropagation de la canne à sucre : application à l'assainissement des plantations. AMADEPA, Journée Canne à Sucre, 1998/07/09, Le Lamentin, Martinique.

24. **Daugrois J.H**., Costet L., Feldmann P., Rott P., 1997. Leaf scald resistance in offspring of *Saccharum spontaneum* clones analysed by pathogen population densities . ISSCT Pathology and Molecular Biology Workshop. Mount Edgecombe, 1997/05, KwaZulu Natal, Afrique du Sud.

25. Feldmann. P., Rott P., Oriol P., Paulet F., **Daugrois J.H**., Sapotille J., 1997.Use of tissue culture of sugarcane for rapid propagation and disease control in Guadeloupe. INICA Congress, 1997/06/23-27, Jovellanos Mayari, Cuba.

26. Feldmann P., **Daugrois J.H**, Oriol P., 1996. L'amélioration génétique de la canne à sucre. AMADEPA., Première journée technique de l'AMADEPA. 1996/04/24, Lamentin, Martinique.

27. Grivet L., D'Hont A., Roques D., **Daugrois J.H**., Paulet F., Glaszmann J.C., 1996. First step towards markings disease resistance and sugar yield component genes in a modern sugarcane cultivar. EUCARPIA., Meeting on Tropical Plants, 1996/03/11-15, Montpellier, France.

28. D'Hont A., Grivet L., Lu Y.H., Roques D., Feldmann P., Rao P.S., **Daugrois J.H.,** Dufour P., Berding N., Walker D.I.T., Hamon P., Glaszmann J.C., 1995. The genome of modern sugarcane varieties. XXII congress, 11th-15th September 1995, Cartagena, Colombia.

29. D'Hont A., Grivet L., Lu Y.H., Roques D., Feldmann P., Rao P.S., **Daugrois** J.H., Dufour P., Berding N., Hamon P., Glaszmann J.C., 1995.The genome of modern sugarcane varieties. Society for Experimental Biology Symposium. 50, 1995/08/29-31, Cambridge, Royaume-Uni.

30. **Daugrois J.H**., Letourmy P., 1995. Quantitative method for studying sugarcane resistance to *Puccinia melanocephala* . XXII congress, 11th-15th September 1995, Cartagena, Colombia.

31. **Daugrois J.H**., 1992. Evaluation rapide de la résistance des variétés de canne à sucre en cours de sélection, à deux maladies cryptogamiques : la rouille et le charbon. Congrès de la Société de Technologie Agricole et Sucrière de Maurice. 9, 1992/10/12-16, Réduit, Maurice

Chapitre 2 : Synthèse des travaux Scientifiques

2.1 Introduction :

J'ai effectué mes premières recherches en phytopathologie à l'Université Paul Sabatier lors de mon stage de DEA et de mes travaux de thèse. Celles-ci m'ont permis d'appréhender la complexité des relations plante-hôte pathogène lors d'étude des interactions entre *Phaseolus vulgaris* et *Colletotrichum lindemuthianum*.

Après ma thèse, j'ai été recruté en 1990 par le CIRAD (Centre de coopération International en Recherche Agronomique pour le Développement) en tant que phytopathologiste au sein du programme canne à sucre. J'ai été affecté d'abord à la Réunion, puis en Guadeloupe.

A la Réunion, j'ai développé des tests de résistance de la canne à sucre aux maladies en appui à la sélection locale avec une activité de recherche sur la résistance de la canne à sucre à la rouille, maladie cryptogamique provoquée par *Puccinia melanocephala*.

En Guadeloupe, j'ai poursuivi, entre 1993 et 2000, mes travaux sur la sélection de variétés résistantes aux maladies majeures en appui à l'équipe de création variétale. Mes activités de recherche ont porté sur deux maladies vasculaires de la canne à sucre que sont l'échaudure des feuilles et le rabougrissement des repousses, provoquées par deux bactéries, respectivement *Xanthomonas albilineans* et *Leifsonia xyli* subsp. *xyli*. Ces études ont concerné essentiellement la dynamique des populations in planta, l'impact de la maladie sur la production, la variabilité de la pathogénie et la recherche de sources de résistance. En 1996, j'ai débuté des études sur une maladie émergeante : la maladie de la feuille jaune dont l'agent causal est un virus de la famille des *Luteoviridae* nommé SCYLV ou '*sugarcane yellow leaf virus'*.

Entre 2000 et 2001 j'ai effectué un séjour scientifique de un an à l'université de Louisiane à Baton Rouge, USA, pour étendre mes compétences aux maladies telluriques de la canne à sucre notamment en développant une étude sur la maladie de pourriture des racines provoquée par *Pythium* sp.

Depuis 2001, j'ai repris mes travaux en Guadeloupe avec une orientation de mes recherches vers l'épidémiologie et la dynamique de deux agents pathogènes de la canne à sucre, *X. albilineans* et SCYLV.

Les recherches effectuées dans des contextes géographiques différents m'ont permis de mieux appréhender la complexité et la diversité des champs disciplinaires nécessaires à la compréhension des complexes parasitaires. Elles ont eu pour objectifs :

- de comprendre les phénomènes liés à l'infection d'une plante par un pathogène,
- d'identifier et caractériser les maladies parasitaires et appréhender leur effet sur les cultures, en l'occurrence la canne à sucre,
- d'appréhender les contraintes externes à la plante pouvant influencer le développement des maladies,
- d'identifier les mécanismes et sources génétiques de résistance.

Ces différents points seront abordés dans la synthèse de mes travaux de recherches menées :

- à l'université de Toulouse sur l'anthracnose du haricot,
- à l'université de Baton Rouge sur une maladie tellurique de la canne à sucre : la maladie de pourriture des racines,
- au Cirad sur trois des principales maladies de la canne à sucre : la rouille, l'échaudure des feuilles et la maladie de la feuille jaune.

2.2 L'anthracnose du haricot : les β-1,3-glucanases du haricot, leur implication dans la résistance à l'anthracnose.

L'objectif de ce travail était d'approcher le rôle d'enzymes hydrolytiques (7), présentant une activité β-1,3-glucanase, dans le système de défense du haricot contre le champignon responsable de l'anthracnose, *Colletotrichum lindemuthianum*. Ces enzymes sont connues pour leur rôle dans diverses interactions plantes pathogènes (26, 28). Disposant de plusieurs races du champignon et de cultivars de haricots sensibles ou résistants à ces diverses races, j'ai étudié les variations quantitatives et qualitatives des β-1,3-glucanases présentes dans les limbes de haricots lors d'interaction hôte-parasite de type compatible et incompatible.

L'étude comparative des plantes saines et inoculées avec 2 lignées isogéniques de haricot (*Phaseolus vulgaris*) ne différent que par un seul gène de résistance à certaines race de *C. lindemuthianum* m'a permis de caractériser :

1/ trois β-1,3-glucanases constitutives dont deux ont des activités endolytiques ainsi que des pI (point isoélectrique) acides. La troisième est une exo-glucanase, possédant aussi une activité β-glucosidase. Celle-ci a un pI basique. Ces enzymes constitutives sont, du moins pour une part, situées au niveau des structures apoplastiques ; elles peuvent à des degrés divers, hydrolyser, *in vitro*, les parois du champignon. Le rôle de ces enzymes dans le système de défense de la plante reste en partie inconnu. Toutefois elles pourraient, par leur action, ralentir la progression du champignon pathogène lors des premières phases de l'infection et contribuer au déclenchement de systèmes de défense en libérant des éliciteurs fongiques lors de leur action sur les parois du champignon.

2/ deux nouvelles formes de β-1,3-glucanase, produites suite à l'inoculation, non présentes dans la plante saine. Ces enzymes ont été totalement purifiées et certaines de leurs propriétés étudiées. Il s'agit d'endo-β-1,3-glucanases de pI basique et de faible poids moléculaire. Le séquençage réalisé pour ces deux protéines confirme qu'elles sont codées par deux gènes différents.

Deux β-1,3-glucanases basiques ont donc été décelées, pour la première fois chez le haricot, suite à une inoculation ou à un stress. Ces deux enzymes ont des caractères immunologiques communs avec les 'PR' (pathogenesis related) protéines à activité glucanase d'autres plantes (19, 18). Ces deux enzymes sont responsables de l'augmentation de l'activité glucanase observée lors de l'inoculation. Cette stimulation est toujours plus précoce lors de l'interaction incompatible (résistance) que lors de l'interaction compatible (sensible). Nous avons déclenché la stimulation de ces deux enzymes en l'absence du pathogène par des éliciteurs fongiques (endoPG et fragments de parois mycéliennes). Une, au moins, des deux enzymes élicitées peut, *in vitro*, hydrolyser les parois du champignon pathogène et une des deux enzymes a été localisée dans les structures apoplastiques. Compte tenu de ces observations, ces enzymes pourraient participer à l'inhibition de la croissance des hyphes infectieux du pathogène, voire à leur destruction.

Les travaux réalisés ont permis de clarifier le rôle des β-1,3-glucanases dans l'interaction plante/pathogène.

Travaux publiés dans :

Daugrois J.H., Lafitte C., Barthe J.P., Faucher C., Touzé A., Esquerre Tugaye M.T., 1992. Purification and characterization of two basic Beta-1,3-glucanases induced in *Colletotrichum lindemuthianum* infected bean seedlings. Archives of Biochemistry and Biophysics, vol. 292, n. 2, p. 468-474.

Daugrois J.H., Lafitte C., Barthe J.P., Touzé A., 1990. Induction of Beta-1,3-glucanase and chitinase activity in compatible and incompatible interactions between *Colletotrichum lindemuthianum* and bean cultivars. Journal of Phytopathology, vol. 130, n. 3, p. 225-234

2.3 Etudes des maladies telluriques de la canne à sucre : effet de nouvelles formulations d'herbicide sur la maladie de pourriture des racines de la canne à sucre.

C'est dans le cadre d'un projet de coopération scientifique avec le laboratoire du Dr Jeffrey Hoy de l'Université de Louisiane à Baton Rouge, que j'ai effectué mes recherches sur les agents pathogènes telluriques de la canne à sucre du genre *Pythium*. Le thème de recherche développé pendant mon séjour a concerné l'étude de l'effet d'herbicides, inhibiteurs de protoporphyrinogène oxydase (PPOase), sur des agents telluriques du genre *Pythium* dont la pourriture de racine de la canne à sucre causée par *Pythium arrhenomanes* (23). Si les PPOase sont une nouvelle famille d'herbicide récemment testée sur la canne à sucre (40) leur impact sur les pourritures racinaires est inconnu alors que d'autres herbicides sont connus pour augmenter ces même pourritures des racines (11). En revanche, sur d'autres cultures que la canne à sucre, des herbicides inhibiteurs de PPOase semblent réduire l'effet de maladies fongiques (22, 6). Du fait de la complexité de l'expression des maladies telluriques de la canne à sucre, de la possibilité d'interaction entre microorganisme du sol et du potentiel d'impact des herbicides sur la microflore du sol (15) nous avons aussi examiné l'impact des herbicides inhibiteurs de PPOase sur la microflore du sol. Ceci a été réalisé en étudiant l'effet des traitements herbicides sur les profils d'utilisation de sources carbonées par la communauté microbienne du sol (14)

Les herbicides testés (azafenidin, flumioxazin et sulfentrazone) lors de ce travail inhibent fortement la croissance de différentes espèces de *Pythium in vitro* dont *Pythium arrhenomanes*. Deux de ces trois herbicides, appliqués sur la surface des feuilles, ont réduit les symptômes de pourriture de racine et semblent avoir eu un effet positif sur la croissance des plantes. On a aussi observé une variation du profil d'activité de la microflore du sol suite aux applications des herbicides sur le sol, notamment pour ce qui concerne les sources carbonées de type carbohydrates. Ces mêmes carbohydrates permettent de différencier les profils microbiens du sol sous différents système de culture à base de pomme de terre, systèmes ayant une influence sur les maladies d'origine tellurique (21). L'impact des herbicides sur la microfaune du sol, sous culture de canne à sucre, peut donc jouer un rôle dans les relations agent pathogène tellurique – plante d'autant plus que la diversité bactérienne de la rhizosphère peut avoir un impact sur la bonne tenue des cultures de canne à sucre (29).

Travaux publiés dans :

Daugrois J.H., Hoy J.W. and J.L. Griffin, 2004. Protoporphyrinogen oxidase inhibitor herbicide effects on Pythium root rot of sugarcane. Phytopathology, vol.95:n°3, p. 220-226.

2.4 Etudes des maladies majeures affectant le système 'aérien' de la canne à sucre :

Les recherches effectuées au CIRAD dans les différentes localisations ainsi que les missions d'expertise m'ont permis d'appréhender la particularité, la diversité et l'importance des maladies parasitaires du système aérien chez une plante complexe qu'est la canne à sucre.

En effet, la canne à sucre (*Saccharum* spp.) est une monocotylédone de la famille des poacées, de la tribu des Andropogonées et appartient au genre *Saccharum*. La canne à sucre fournit plus des deux tiers de la production mondiale de sucre et est une des plantes les mieux adaptées pour la production de bioéthanol. Elle constitue la source exclusive de sucre pour les pays en développement. Dans ces pays, la consommation augmente régulièrement et la

satisfaction des besoins est souvent une priorité nationale. La recherche menée au CIRAD contribue à augmenter la production en améliorant la productivité et la qualité, dans des conditions climatiques et socioéconomiques très diversifiées.

Comme pour beaucoup de plantes, les maladies constituent des facteurs limitants importants de la production de sucre. Plus de 60 maladies d'origines diverses sont répertoriées pour la canne à sucre (1). Pour lutter contre ces maladies, aucun traitement sanitaire n'est pratiqué dans la plupart des zones de production tout comme en Guadeloupe. De plus, la propagation des variétés (clones) s'effectue par boutures et le système de culture est pluriannuel (4 à 10 ans), entre chaque cycle de plantation. Ces caractéristiques propres à la canne à sucre en font un modèle d'étude quasiment unique mais très diversifié car présent sur plus de 18 millions d'hectares répartis dans 82 pays.

La lutte contre les maladies de la canne à sucre peut se résumer à l'utilisation de variétés résistantes (41) et la production de plants de bonne qualité sanitaire pour la plantation (12, 13). La sélection de variétés résistantes aux maladies, présentes et à venir, est donc primordiale pour la pérennisation de la culture. Cette sélection nécessite une bonne connaissance des maladies et de leur agent pathogène. En revanche, si nombre de travaux ont été réalisés sur les maladies de la canne à suce, les études génétiques de la résistance de la canne à sucre aux maladies ont longtemps été limitées par la complexité de l'organisation de son génome. En effet, les variétés modernes de canne à sucre ont une structure génétique très complexe. Elles sont hautement polyploïdes et aneuploïdes et résultent de croisements interspécifiques entre deux espèces du genre *Saccharum*. La première est l'espèce domestiquée, productrice de sucre mais sensible à de nombreuses maladies, *Saccharum officinarum* (2n = 8x = 80) et la deuxième est l'espèce sauvage *Saccharum spontaneum* (2n = 5x – 16x = 40 – 128). Les variétés modernes possèdent 100 à 130 chromosomes, dont environ 15 à 25 % proviennent de l'espèce sauvage (10).

Mes recherches menées au CIRAD à la Réunion puis en Guadeloupe tiennent compte de ces particularités de la canne à sucre et tendent à favoriser la création et la sélection de variétés résistantes aux maladies. Elles ont concernées trois des principales maladies de la canne à sucre : la rouille (agent causal : *Puccinia melanocephal*a), l'échaudure des feuilles (agent causal : *Xanthomonas albilineans*) et la maladie de la feuille jaune (agent causal : *Sugarcane Yellow Leaf Virus*).

2.4.1 La rouille de la canne à sucre : identification d'un gène majeur de résistance.

La rouille commune de la canne à sucre est causée par un champignon basidiomycète, *Puccinia melanocephala* H. & P. Sydow. Cette maladie est répartie dans l'ensemble des régions du Monde cultivant la canne à sucre. Le caractère de résistance à cette maladie est connu pour être héréditaire (17, 39). Les symptômes de la maladie sont aisément reconnaissables ce qui facilite la sélection et la création de variétés résistantes. En revanche, la quantification de la résistance nécessite des données reproductibles et comparables. Les symptômes notés pour évaluer les clones correspondent à un dénombrement des lésions (urédinia) observées par unité de surface. Ces données sont de type agrégatif et difficilement utilisables pour une comparaison directe des résultats. Afin de faciliter l'interprétation de ces données, nous avons décrit un modèle permettant, après transformation des données, une analyse paramétrique des observations et une comparaison de la résistance des variétés.

Les études menées pour normer la lecture des symptômes et le classement des variétés nous ont permis d'identifier un gène majeur de résistance à la rouille chez la canne à sucre. Ce gène a été identifié lors de l'analyse de la résistance à la rouille d'une descendance issue d'une autofécondation de la variété R570. Malgré la complexité génétique de la canne à sucre, une

ségrégation 3:1 a été clairement mise en évidence après infection des plants en conditions naturelles et en conditions contrôlées. Cette même descendance a été utilisée avec la collaboration du laboratoire Biotrop du Cirad Montpellier pour effectuer une cartographie génétique, à l'aide de marqueurs RFLP, du génome de R570. Le gène de résistance a pu être localisé à moins de 10 cM d'un marqueur RFLP (500kb/cM).

Ces résultats réfèrent pour la première fois à un caractère à déterminisme mono-génique chez une plante hautement polyploïdie comme la canne à sucre. Si ce modèle de déterminisme simple de résistance pouvait s'appliquer à d'autres maladies pour lesquelles l'évaluation de la résistance des clones est très laborieuse (comme pour l'échaudure des feuilles, ou d'autres maladies insidieuses) l'utilisation de marqueurs moléculaires serait alors profitable aux programmes de création variétale pour la sélection de variétés résistantes.

Depuis son identification, la position du gène a été affinée (3) et une collaboration internationale a été mise en place pour cloner ce gène de résistance à la rouille.

Travaux publiés dans :

Daugrois J.H., Letourmy P., 1996. Quantitative method for studying sugarcane resistance to *Puccinia melanocephala*. In : Proceedings XXII congress, 11th-15th September 1995, Cartagena, Colombia. Vol. 2. Cal, Colombie, Tecnicana, p.548-556

Daugrois J.H., Grivet L., Roques D., Hoarau J.Y., Lombard H., Glaszmann J.C., D'Hont A., 1996. A putative major gene for rust resistance linked with a RFLP marker in sugarcane cultivar R570. Theoretical and Applied Genetics, vol. 92, p. 1059-1064.

D'Hont A., Grivet L., Lu Y.H., Roques D., Feldmann P., Rao P.S., Daugrois J.H., Dufour P., Berding N., Walker D.I.T., Hamon P., Glaszmann J.C., 1996. The genome of modern sugarcane varieties. In : International society of sugarcane technologists. Proceedings XXII congress, volume 2 : agriculture biology. Cali, Colombia, TECNICA, p.363-367.

2.4.2 Etude de l'échaudure des feuilles : dynamique, variabilité de l'agent causal et sources de résistance des plantes à cette maladie.

Xanthomonas albilineans est l'agent causal de l'échaudure des feuilles de la canne à sucre, maladie présente en Guadeloupe et dans la majorité des aires de culture de cette plante. De nombreuses recherches ont été menées depuis longtemps sur l'échaudure des feuilles. Si certains aspects de la maladie sont bien connus et décrits (34), tels que l'expression des symptômes qui peuvent apparaître sur les plants malades, ou encore le rôle de la toxine (albicidine) dans cette symptomatologie, d'autres aspects sont encore méconnus.

Présente dans plus de 60 pays, elle demeure une des maladies majeures de la canne à sucre non seulement à cause de son effet sur la production de sucre et sur la perte de variété en collection ou lors des processus de sélection, mais aussi à cause de sa phase de latence. Les plantes peuvent être infectées sans pour autant montrer de symptômes. Une autre caractéristique de la maladie est l'observation d'épidémie sporadique.

Si l'échaudure des feuilles est connue depuis longtemps pour être transmise par les outils de coupe et par l'utilisation de boutures infectées (30), de nombreuses observations, notamment la présence de bactéries dans les gouttelettes de gutation, suggèrent la possibilité d'une contamination des plantes par voie aérienne (20, 36). L'échaudure des feuilles est causée par une bactérie, *X. albilineans*, envahissant le xylème de la plante. Cette bactérie se différencie facilement d'autres bactéries du même genre. En revanche, on observe une certaine hétérogénéité

à l'intérieur de cette espèce (9). Hétérogénéité qui peut jouer un rôle dans les variations d'expression de la maladie.

Les recherches que j'ai menées en Guadeloupe ont été développées pour mieux comprendre le cycle infectieux de la maladie afin d'en renforcer la lutte, soit par la création de variétés résistantes, soit par la production de plants assainis. Ces études ont concerné l'épidémiologie, la variabilité de l'agent pathogène et la résistance du complexe *Saccharum spp*. à l'échaudure des feuilles.

Epidémiologie :

Le suivi de la dynamique des populations épiphytes de *X. albilineans* sur des parcelles de canne à sucre mises en place avec des plants sains, issus de culture *in vitro,* m'a permis de mettre en évidence, pour la première fois, le fait que la contamination de la phyllosphère de la canne à sucre puisse être un préalable à la contamination des plantes elles-mêmes. La contamination de la phyllosphère a été appréhendée par la mesure des populations bactériennes de *X. albilineans*, dans les gouttes de rosée ou de pluie, présentes à la surface des feuilles le matin. *X. albilineans* a été identifié dans ces eaux de surface et de fortes populations ont pu être dénombrées préalablement à l'observation de symptômes nécrotiques sur les feuilles. Ces observations laissent supposer que la phase épiphyte de *X. albilineans* joue un rôle important dans le cycle infectieux de la maladie. Toutefois, l'agressivité de la souche contaminant la phyllosphère et la densité de population de *X. albilineans* présente à la surface des feuilles sont déterminantes pour la contamination des plantes suite à l'installation des populations épiphytes de *X. albilineans*. En effet les souches les plus virulentes sont plus aptes à pénétrer les tissus foliaires et favorisent ainsi les phénomènes d'ingression et d'égression entre la surface et les tissus internes de la plante (4, 16). Ces processus sont favorables au développement de fortes populations bactériennes dans l'eau, à la surface des feuilles, et consécutivement, à la dispersion de l'agent pathogène de plante à plante.

Si la colonisation de la phyllosphère de la canne à sucre par *X. albilineans* est une étape importante du cycle infectieux de la maladie de l'échaudure des feuilles en Guadeloupe, en revanche, l'impact du niveau de résistance de variétés de canne à sucre sur la colonisation de la phyllosphère et la dynamique des populations bactériennes épiphytes de *X. albilineans* au cours de cycles de récolte successifs est inconnue. Afin de répondre à cette problématique, nous avons mesuré les populations épiphytes de *X. albilineans* pendant 3 cycles de récolte à la surface des feuilles de deux variétés de canne à sucre qui diffèrent par leur niveau de résistance à l'échaudure des feuilles (32). Après transfert au champ de plants sains de canne à sucre, issus de culture *in vitro*, nous avons mesuré les populations bactériennes de *X. albilineans* régulièrement dans les gouttes de rosée prélevées à la surface des feuilles de canne à sucre. Après le début de colonisation de la canopée par *X. albilineans*, la densité des populations bactériennes moyennes a progressé plus rapidement sur les feuilles de la variété sensible que sur celles de la variété résistante. Néanmoins, en fin de colonisation de la phyllosphère de la canne à sucre, les densités de populations épiphytes moyennes de *X. albilineans* observées dans les gouttes de rosée étaient similaires pour les 2 variétés étudiées. La présence de l'agent pathogène dans les tiges de canne à sucre a été contrôlée par isolement des bactéries à chaque cycle de récolte, après 11 à 12 mois de croissance des plantes. La proportion de tiges infectées par *X. albilineans* a varié en fonction du niveau de résistance des variétés et, pour la variété sensible, en fonction des densités de populations épiphytes de l'agent pathogène. L'importance des densités de populations bactériennes épiphytes est donc primordiale, mais non suffisante, dans le processus de contamination des tiges après transmission par voie aérienne de l'agent pathogène de l'échaudure des feuilles.

La vie épiphyte de *X. albilineans* est donc une réalité, du moins dans les conditions tropicales humides de la Guadeloupe. Cette phase épiphyte est une clé importante du cycle infectieux de cette maladie, notamment pour les variétés sensibles, et est fortement influencée par les conditions climatiques locales. Dans ces conditions, l'utilisation d'un schéma de multiplication ex situ de plants assainis (pépinières) ne permet pas une lutte efficace contre cette maladie si cette lutte n'est pas accompagnée par une sélection de variétés résistantes.

Travaux publiés dans :

Daugrois J.H., Dumont V., Champoiseau P., Costet L., Boisne-Noc R., Rott P., 2003. Aerial contamination of sugarcane in Guadeloupe by two strains of *Xanthomonas albilineans*. European journal of plant pathology, vol.109, p. 445-458.

Daugrois J.H., Champoiseau P., Boisne-Noc R., Rott P, 2005. Epiphytic colonization and infection by *Xanthomonas albilineans* of two sugarcane cultivars differing in resistance to leaf scald disease. Proceedings of XXV ISSCT congress, Guatemala City, Guatemala, 2005, p678-685.

Daugrois J.-H., Champoiseau P., Rott P., 2006. Impact of rainfall on epiphytic colonization of sugarcane by the leaf scald pathogen and associated plant infection. VIIIth ISSCT Pathology Workshop Petit-Bourg, Guadeloupe (FWI), 23 - 27 January 2006

Variabilité de l'agent pathogène :

La pérennité de la résistance des variétés cultivées peut être menacée par l'existence de variabilité phénotypique et génétique de l'agent causal. En effet l'agent pathogène qu'est *X. albilineans* peut montrer une variabilité d'agressivité entre différentes souches même si elles sont issues d'une même zone géographique (27). Cette diversité d'agressivité, étudiée sur des souches de Guadeloupe, a conclu sur l'existence de pathotypes, plus ou moins agressifs, plutôt que l'existence de races.

Associer cette variabilité d'agressivité à un ou des caractères génétiques de l'agent pathogène permettrait d'identifier les gènes liés à la pathogénie de *X. albilineans*, pathogénie qui semble différente d'autres bactéries pathogènes, y compris du genre *Xanthomonas*. En effet, pour nombre de bactéries Gram- négatives, pathogènes des plantes et des animaux, la pathogénie est liée à la présence du système de sécrétion type III qui permet aux bactéries pathogènes d'injecter dans la cellule de l'hôte des protéines effectrices particulières à chaque agent pathogène (8). Ce système de sécrétion type III n'a pu être identifié chez *X. albilineans* laissant supposer que d'autres mécanismes liés à la pathogénie existent. En revanche, *X. albilineans* produit une toxine, l'albicidine, qui joue un rôle clé dans la pathogénie de cette bactérie (5). La description et le séquençage récent des gènes impliqués dans la production de cette toxine (35) nous fournissent la première matière pour étudier la variation de gènes liés à la pathogénie de *X. albilineans*. Si, à partir de 63 souches de *X. albilineans* prélevées en Guadeloupe entre 1999 et 2002, nous avons pu observer des variations de production d'albicidine *in vitro*, aucune variation quand aux gènes impliqués dans la synthèse de cette toxine n'a été mise en évidence pour les souches issues de Guadeloupe, alors que pour des souches de *X. albilineans* isolée à travers le monde, 14 haplotypes et 2 groupe génétiques ont été identifiés. De plus aucun lien entre diversité des gènes de biosynthèse de l'albicidine, variation de production d'albicidine et variation de l'agressivité des souches n'a pu être mis en évidence.

Ces résultats permettent de supposer que d'autres gènes sont impliqués dans l'expression du pouvoir pathogène de *X. albilineans*.

Afin de rechercher les gènes impliqués dans l'expression du pouvoir pathogène et d'élargir la base d'étude de *X. albilineans*, une collection regroupant 147 souches de *X. albilineans*, prélevées dans la plus part des zones géographiques cannière de l'archipel, a été constituée. Ces 147 souches appartiennent au même sérotype parmi les 3 identifiés chez *X. albilineans* (31) et ont été testées pour leur capacité à produire de l'albicidine. Soixante quinze souches ont été choisies sur la base de leur diversité de production d'albicidine et de leur origine géographique dans l'archipel. Ces 75 souches ont fait l'objet d'analyse de pathogénie sur la base de leur capacité à coloniser la canne à sucre après inoculation et à produire des symptômes sur une variété sensible. Les variations observées ont été confirmées sur 19 souches représentatives de la distribution de la pathogénie des 75 souches. Ces travaux ont permis d'identifier 4 groupes de pathogénie différente.

Des variations génétiques ont pu être identifiées par AFLP, à l'aide de 16 couples d'amorces, sur les 9 souches appartenant à 4 groupes de pathogénie différente. Toutefois ces variations génétiques n'ont pas pu être reliées aux variations de pathogénie. De la même façon nous avons recherché chez *X. albilineans* la présence de 40 gènes impliqués dans la pathogénie d'espèces bactériennes proches. Seulement 3 de ces gènes ont pu être identifiés chez *X. albilineans*, et ils sont présents et de séquence homologue chez les 9 souches de pathogénie différente. L'origine de la variation de l'agressivité de souches proches génétiquement demeure inconnue. Toutefois seule une faible part du génome a été étudiée en détail. Du fait qu'aucune modification majeur du génome n'a été détectée, il est probable que des mutations nucléotidiques simples de un ou plusieurs gènes impliqués dans la pathogénèse de *X. albilineans* sont responsables des variations de pathogénie observées. L'étude de la séquence complète du génome de *X. albilineans* devrait nous permettre par comparaison avec d'autres bactéries phytopathogènes d'identifier les gènes de pathogénies et de caractériser les mécanismes moléculaires impliqués dans l'interaction du couple canne à sucre *X. albilineans*. Actuellement, la séquence complète du génome d'une souche de *X. albilineans* issue de cette étude est en cours au Genoscope.

Travaux publiés dans :

Daugrois J.H., Champoiseau P., Boisne-Noc R., Rott P., 2000. Variability in pathogenicity of *Xanthomonas albilineans* in Guadeloupe. Phytopathology, vol. 90 (6): S18.

Champoiseau, P.; Daugrois, J.-H.; Girard, J.-C.; Royer, M.; Rott, P., 2006. Variation in albicidin biosynthesis genes and in pathogenicity of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. Phytopathology, vol.96 (1): 33-45

Champoiseau, P., Daugrois, J.-H., Pieretti, I., Cociancich, S., Royer, M. and Rott, P. 2006. High variation in pathogenicity of genetically closely related strains of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen, in Guadeloupe. Phytopathology, vol.96 (10):1081-1091.

Résistance des plantes à l'échaudure des feuilles :

L'analyse de la résistance des clones de canne à sucre était à l'origine basée sur l'observation des symptômes foliaires. Néanmoins, les plantes peuvent être contaminées sans montrer de symptômes (phase de latence) (34). Pour cette raison, l'analyse de la résistance des clones a été abandonnée par plusieurs centres de recherche. En revanche, il a été démontré que la limitation de la progression de *X. albilineans* dans la tige est une caractéristique majeure de la résistance à l'échaudure des feuilles (33). C'est sur cette base que les techniques d'évaluation de la résistance des variétés de canne à sucre à l'échaudure des feuilles ont été modifiées en Guadeloupe. La résistance des clones est maintenant analysée en évaluant les populations de

pathogènes présentes dans le xylème au niveau de la tige, après inoculation ou infection naturelle, tout en tenant compte des symptômes lorsqu'ils apparaissent.

Nous avons utilisé ces nouvelles techniques pour rechercher des sources de résistances chez 56 accessions issues de 5 espèces apparentées à la canne à sucre, *S. barberi*, *S. officinarum*, *S. robustum*, *S. sinense* et *S. spontaneum*. Ces études ont permis d'identifier un potentiel de sources de résistance chez les clones de l'espèce sauvage *S. spontaneum*.

Si parmi les accessions testées, les clones appartenant à l'espèce *S. spontaneum* sont les plus résistants quant à la colonisation des tiges par l'agent pathogène, la transmission de cette résistance semble complexe. En effet, la capacité de transmettre cette résistance, étudiée sur trois descendances issues d'autofécondations de 3 accessions de l'espèce *S. spontaneum*, varie en fonction du clone utilisé comme géniteur. Pour 2 descendances nous avons montré que les moyennes des densités de population bactérienne, mesurées dans la tige, sont équivalentes à la valeur du parent, dont ces descendances sont issues. Pour ces 2 accessions les résultats indiquent donc une bonne transmission du caractère de résistance. En revanche, pour la troisième descendance étudiée, nombre de clones se sont avérés plus sensibles que le parent. Pour l'ensemble des clones de cette descendance, la moyenne des populations bactériennes mesurées dans les tiges était 50 fois supérieure à celle obtenue pour le parent. Pour ce clone on peut émettre l'hypothèse que le caractère de résistance ne se trouve que sur une partie des copies du génome et, par conséquent, non transféré ou transféré que pour partie à sa descendance.

Le caractère de résistance à l'échaudure des feuilles semble donc beaucoup plus complexe génétiquement que le caractère de résistance à la rouille décrit précédemment. Quoiqu'il en soit certains clones de l'espèce *S. spontaneum* peuvent être utilisés comme source de résistance à l'échaudure des feuilles.

Travaux présentés à :

Daugrois J.H., Costet L., Feldmann P., Rott P., 1997. Leaf scald resistance in offspring of *Saccharum spontaneum* clones analysed by pathogen population densities. In: SASA., International society of sugar cane technologists. Abstracts of the pathology and molecular biology workshop. Mount Edgecombe, Afrique du Sud, SASA.

Daugrois J., Costet L., Rott P., 1998. Resistance to leaf scald disease in wild relatives of sugarcane analysed by pathogen population densities. In: BSPP, ISPP., *ICPP98. Offered papers. Abstracts (themes 3, 4, 5 and miscellaneous)*. Birmingham, Royaume-Uni, BSPP, vol. 3, p. 3.4.7. International Congress of Plant Pathology. 7, 1998/08/09-16, Edinburgh, Royaume-Uni.

2.4.3 Etude de la maladie de la feuille jaune : de son identification à la caractérisation du cycle infectieux et des moyens de lutte.

Le syndrome des feuilles jaunes dont l'étiologie correspond à la maladie de la feuille jaune a été observé pour la première fois en Guadeloupe en 1994. Le virus responsable de cette maladie, le *sugarcane yellow leaf virus* (SCYLV) a été identifié en Guadeloupe et Martinique en 1996, comme dans d'autres pays producteurs de canne à sucre (38).

L'agent pathogène

Le SCLYV est un *Polerovirus* de la famille des *Luteoviridae*. La particule virale mesure 24 à 26 nm de diamètre et son génome est constitué d'un ARN simple brun (24). Elle envahit exclusivement les vaisseaux du phloème de la plante. Ce virus est transmis à la plante par des pucerons notamment *Melanaphis sacchari* et *Ropalosiphum maidi* (37). Ces 2 pucerons sont

présents en Guadeloupe, mais c'est essentiellement le premier que l'on retrouve sur les plants de canne à sucre. Nous avons observé que les plantes fortement atteintes présentent un jaunissement foliaire prématuré notamment au niveau de la partie médiane de la feuille comme décrit par ailleurs (25). Toutefois, si le virus n'est pas responsable de tous les jaunissements foliaires observés, sa présence dans la plante favorise l'expression de ces symptômes.

A partir de différents isolats de SCYLV des variabilités génomiques ont été observées lors de séquençages génétiques obtenus après traduction partielle ou complète du génome viral. Quatre groupes génétiques différents ont été identifiés après analyse phylogénique des séquences obtenues (2). Ces groupes génétiques ont été nommés en fonction de l'origine de la souche virale (BRA-Brésil-, PER-Pérou-, REU-Réunion- et CUB-Cuba-). La définition d'amorces spécifiques a été effectuée afin de distinguer par diagnostic RT-PCR ces quatre génotypes. Seul BRA et PER, proches génétiquement, n'ont pu être distingué l'un de l'autre et seront considérés comme un seul génotype BRA-PER dans nos études. Ces amorces spécifiques nous ont permis d'identifier les génotypes présents en Guadeloupe et dans différentes zones géographiques. La Guadeloupe reste actuellement la seule zone ou les trois génotypes BRA-PER, REU, CUB ont été identifiés et transmis naturellement par le vecteur sur des variétés locales. Le nombre élevé de variétés testées et d'échantillons analysés peut être la cause de ce diagnostic.

Quoiqu'il en soit, la présence de différents groupes génétiques de cet agent pathogène pose le problème d'éventuelles variations de l'agressivité et du potentiel de dispersion lié à la variabilité génétique identifiée. Une étude comparative, sur des souches issues de La Réunion et de Guadeloupe concernant l'aptitude de la souche virale (génotype) à la transmission et la réponse de la plante en fonction de son statut (résistante, sensible), nous a permis de mettre en évidence i/ des variations dans la capacité des différents génotypes à contaminer certaines variétés, ii/ des variations de virulence entre les génotypes étudiés (BRA-PER, REU, CUB).

Afin de mieux appréhender l'importance les risques liés à cette maladie nous étudions actuellement la répartition et la fréquence du virus et de ses divers génotypes sur la sole cannière de Guadeloupe et Martinique. Les premiers résultats obtenus montrent que les taux d'infection des plantes par le virus varient de 0 à 40% en Guadeloupe et semble dépendre de la zone géographique concerné, de la variété considérée et du nombre de cycles de la culture. Le génotype REU à été identifié dans toutes les zones infectées en Guadeloupe alors que les 2 autres génotypes sont présents de façon minoritaire dans certaines des parcelles infectées. La présence majoritaire d'un seul génotype permet de supposer que ce génotype est mieux adapté aux conditions environnementales de Guadeloupe (y compris le vecteur) ou que les deux autres génotypes sont d'introduction plus récente et n'ont pas finalisé leur dispersion.

Travaux publiés dans :

Daugrois J.H., Jean-Baptiste I., Lockhart B.E.L., Irey S., Chatenet M., Rott P., 1999. First report of sugarcane yellow leaf virus in French West Indies. Plant Disease, vol. 83, n. 6, p. 588.

Daugrois J.H., Jean-Baptiste I., Rott P., 1999. Partial association of sugarcane yellow leaf syndrome symptoms and sugarcane yellow leaf virus in the French West Indies. In : ISSCT, Proceedings XXIII Congress. ISSCT Congress. 23, 1999/02/21-26, New-Delhi, Inde. pp 401-403.

Abu Ahmad Y., Royer M., Daugrois J.-H., Costet L., Lett J.-M., Victoria J. I., Girard J.-C., and Rott P., 2006. Geographical distribution of four Sugarcane yellow leaf virus genotypes. Plant Dis. 90:1156-1160.

Abu Ahmad Y., Costet L., Daugrois J.H., Nibouche S., Letourmy P., Girard J.C., and Rott P. 2007. Variation in infection capacity and in virulence exists between genotypes of sugarcane yellow leaf virus. Plant Disease, 91: 253-259.

Edon-Jock C., Rott P., Vaillant J., Fernandez E., Girard J.C. and Daugrois J.H. (2007) Status of sugarcane yellow feal virus in commercial fields and risk assessment in Guadeloupe. In : ISSCT, Proceedings XXVI Congress. ISSCT Congress. 26, 2007/07/29-08/02, Durban, South Africa..

Epidémiologie, modèle de dispersion :

La lutte contre cette maladie, hormis la sélection de variétés résistantes, nécessite de limiter la contamination des plants lors de la multiplication des plantes à usage de boutures destinées aux plantations industrielles. Ceci requiert une meilleure compréhension du développement de la maladie. A cette fin, nous avons mené des études d'épidémiologie pour comprendre les processus de contamination de parcelles plantées avec des plants sains, d'une variété sensible, en fonction de la dynamique des populations de pucerons. Nous avons pu observer que l'apparition de la maladie et son expansion sont liées à de fortes populations de *M. sacchari*.

Afin de déterminer les caractéristiques spatio-temporelles de dispersion de la maladie des études complémentaires ont été menées sur des parcelles plantées avec des plants sains issus de culture *in vitro*. Chaque plant a été régulièrement suivi quand à sa contamination par le SCYLV. Ces études nous ont permis de déterminer un modèle de contamination prenant en compte une phase d'infection primaire liée à l'arrivée de pucerons ailés infectieux sur la parcelle, suivie d'une contamination secondaire de proximité à l'intérieur de la parcelle. La phase de contamination primaire s'arrête lors de la couverture totale du sol par le feuillage. Si la contamination des plantes lors de l'infection primaire semble aléatoire, l'infection secondaire, qui a un pas de distance moyen de moins de 2m, donne un caractère agrégatif à la contamination des plantes au bout de quelques semaines. Ces observations nous ont permis de proposer, en collaboration avec l'UAG, un modèle simulant la contamination d'une parcelle. Toutefois ce modèle reste à améliorer en y introduisant le caractère lié à la variété (résistance à la transmission).

Travaux présentés à :

Daugrois J.H., Bonotto S., Siegwart M., Joseph S., and Rott P., 2004. Spread of Sugarcane yellow leaf virus in a healthy sugarcane field and associated populations of Melanaphis sacchari in Guadeloupe. 44th Annual meeting of American Phytopathology society, Caribbean Division, 24-28 May, Havana, Cuba.

Jacquet O., Edon C., Vaillant J., and Daugrois J.-H., 2006. Statistical study of Spatio-temporal evolution of plant infection by SCYLV in a disease free plot. 15th Annual Meeting of the Caribbean Academy of Sciences, Gosier, Guadeloupe May 21-23, 2006.

Résistance des plantes à la maladie de la feuille jaune :

La technique d'immuno-empreinte de tissus végétal permet de visualiser la présence des agents pathogènes sur des empreintes de tissus effectuées sur des feuilles de nitrocellulose. Ces techniques ont été adaptées pour étudier la répartition du virus dans les vaisseaux du phloème de la partie médiane des feuilles et dans des coupes transversales de tige. Nous avons ainsi mis en évidence une variabilité de la charge virale (nombre de vaisseaux colonisés par le virus) et du nombre de plants infectés en fonction de la variété étudiée. Ces résultats laissent supposer que nous disposons en Guadeloupe de gènes de résistance nécessaires pour lutter contre cette maladie. En revanche, les modes et les mécanismes de résistance sont totalement inconnus.

Un projet est en cours pour étudier la stabilité et la diversité génétique de ce caractère de résistance variétale dans une population non structurée de *Saccharum* spp.

Travaux présentés à :

Daugrois J.H., Boisne-Noc R., Rott P., 2000. Testing sugarcane for resistance to sugarcane yellow leaf virus. ISSCT Sugarcane Pathology Workshop. 6, 2000/07/17-23, Cha-am, Thaïlande.

Références bibliographiques :

1. A Guide to Sugarcane Diseases (2000) P Rott, RA Bailey, JC Comstock, BJ Croft, A Salem-Saumtally (editors). La librairie du Cirad, Montpellier, France.
2. Abu Ahmad Y, Rassaby L, Royer M, Borg Z, Braithwaite KS, Mirkov E, Irey MS, Perrier X, Smith GR and Rott P (2006a) Yellow leaf of sugarcane is caused by at least three different genotypes of Sugarcane yellow leaf virus, one of which predominates on the Island of Réunion. Arch. Virol., 151: 1355-1371.
3. Asnaghi C, Roques D, Ruffel S, Kaye C, Hoarau JY, Telismart H, Girard JC, Raboin LM, Risterucci AM, Grivet L, D'Hont A (2004) Targeted mapping of a sugarcane rust resistance gene (*Bru*1) using bulked segregant analysis and AFLP markers. Theor Appl Genet 108:759–764.
4. Beattie GA and Lindow SE (1995) The secret life of foliar bacterial pathogens on leaves. Annual Review of Phytopathology 33: 145–172 .
5. Birch RG and Patil SS (1987) Correlation between albicidin production and chlorosis induction by *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. Physiol. Mol. Plant Pathol. 30:199-20.
6. Black BD, Russin, JS, Griffin JL and Snow JP (1996) Herbicide effects on Rhizoctonia solani in vitro and Rhizoctonia foliar blight of soybean (Glycine max). Weed Sci. 44:711-716.
7. Boller T (1985) Induction of hydrolases as a defense reaction against pathogen. In Key, J.L., and T. Kosuge (rds), cellular and Molecular Biology of Plant Stress. Alan Riss, New York, pp.247-262.
8. Buttner D and Bonas U (2002) Getting across – bacterial type III effector proteins on their way to the plant cell. EMBO J 21: 5313–5322.
9. Davis MJ, Rott P, Warmuth CJ, Chatenet M and Baudin P (1997) Intraspecific genomic variation within *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. Phytopathology 87: 316–324.
10. D'Hont A, Grivet L, Feldmann P, Rao P, Berding N, Glaszmann JC (1996) Characterisation of the double genome structure of modern sugarcane cultivars (*Saccharum spp*.) by molecular cytogenetics. Mol Gen Genet 250:405–413.
11. Dissanayake N, Hoy JW and Griffin JL (1998) Herbicide effects on sugarcane growth, *Pythium* root rot, and *Pythium arrhenomanes*. Phytopathology 88:530-535.
12. Egan BT and Sturgess OW (1980) Commercial control of leaf scald disease by thermotherapy and a clean seed program. Proceedings International Society of Sugar Cane Technologists 17: 1602–1606.
13. Flynn JL and Anderlini TA (1990) Disease incidence and yield performance of tissue culture generated seedcane over the crop cycle in Louisiana. Journal of American Society of Sugar Cane Technologists 10: 113.
14. Garland JL and Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon source utilization. Appl. Environ. Microbiol. 57:2351-2359.
15. Greaves MP and Malkomes MP (1980) Effects on soil microflora. Pages 223-253 in: Interactions Between Herbicides and the Soil. R. J. Hance, ed. Academic Press, New York.
16. Hirano SS and Upper CD (1993) Dynamics, spread, and persistence of a single genotype of Pseudomonas syringae relative to those of its conspecifics on populations of snap bean leaflets. Applied Environmental Microbiology 59: 1082–1091.
17. Hogarth DM, Ryan CC, Taylor PWJ (1993) Quantitative inheritance of rust resistance in sugarcane. Field Crops Res 34:187–193.
18. Joosten MHAJ and De Wit PJCM, 1989. Identification of several pathogenesis-related proteins in tomato leaves inoculated with Cladosporium fulvum as β-1,3-glucanases and chitinases. Plant Physiol., 89, 945-951.
19. Kauffmann, S, Legrand M., Geoffroy P and Fritig B, 1987. Biological function of "pathogenesis-related" proteins: four PR proteins of tobacco have β-1,3-glucanases activity. Embo J., 6 (11), 3209-3212.
20. Klett P and Rott P (1994) Inoculum sources for the spread of leaf scald disease of sugarcane caused by *Xanthomonas albilineans* in Guadeloupe. Journal of Phytopathology 142: 283–291.
21. Larkin RP (2003) Characterization of soil microbial communities under different potato cropping systems by microbial population dynamics, substrate utilization, and fatty acid profiles. Soil Biol. Biochem. 35:1451-1466.

22. Levene BC, Owen, MDK and Tylka GL (1998) Response of soybean cyst nematodes and soybeans (*Glycine max*) to herbicides. Weed Sci. 46:264-270.
23. Lee YS and Hoy JW (1992) Interactions among *Pythium* species affecting root rot of sugarcane. Plant Dis. 76:735-739.
24. Lockhart BEL, Irey MS and Comstock JC (1996) Sugarcane baciliform virus, sugarcane mild mosaic virus and sugarcane yellow leaf syndrome. Page 134 in: Sugarcane gerplasm conservation and exchange, ACIAR Proceedings No 67. Croft, B.J., Piggin, C.M., Wallis, E.S., and Hoggart, D.M. (eds).
25. Lockhart BEL. and Cronjé CPR (2000) Yellow leaf syndrome. In: A guide to sugarcane diseases. P Rott, RA Bailey, JC Comstock, BJ Croft and A. Saumtally (Eds). La Librairie du Cirad, Montpellier, France pp. 291-295.
26. Mauch F, Hadwiwiger LA and Boller T (1988) Antifungal hydrolase in pea tissue: Purifiation and Characterization of two chitinases and two β-1,3-glucanases differentially regulated during development and in response to fungal infection. Plant Physiol. 87, 325-333.
27. Mohamed IS, Rott P, Davis MJ and Chatenet M (1996) Differentiation of *Xanthomonas albilineans* strains based on multiplication of the pathogen in sugarcane varieties. Proc. Int. Soc. Sugar Cane Technol. 22:486-492.
28. Netzer D, Kritzman U and Chet I. (1979) β-1,3-glucanase activity and quantity of fungus in relation to fusazrium wilt in resistant and susceptible near isogenic lines of muskmelon. Physiol. Plant Pathol. 14, 47-55.
29. Pankhurst CE, Hawke BG, Holt JA, Magarey RC and Garside AL (2000) Effect of rotation breaks on the diversity of bacteria in the rhizosphere of sugarcane and its potential impact on yield decline. Proc. Aust. Soc. Sugar Cane Technol. 22:77-83.
30. Ricaud C and Ryan CC (1989) Leaf scald. In: Ricaud C, Ryan BT, Gillaspie AG and Hughes CG (eds) Diseases of Sugarcane (pp 39–58) Elsevier Publishing Co., Amsterdam.
31. Rott P, Davis MJ and Baudin P (1994) Serological variability of *Xanthomonas albilineans*, causal agent of sugarcane leaf scald disease. Plant Pathol. 43:344-349.
32. Rott P, Mohamed IS, Klett P, Soupa D, de Saint-Albin A, Feldmann P and Letourmy P (1997) Resistance to leaf scald disease is associated with limited colonization of sugarcane and wild relatives by Xanthomonas albilineans. Phytopathology 87: 1202–1213
33. Rott P, Mohamed IS, Klett P, Soupa D, de Saint-Albin A, Feldmann P and Letourmy P (1997) Resistance to leaf scald disease is associated with limited colonization of sugarcane and wild relatives by *Xanthomonas albilineans*. Phytopathology 87:1202-1213.
34. Rott P and Davis MJ (2000) Leaf scald. In: Rott P, Bailey RA, Comstock JC, Croft BJ and Saumtally AS (eds) A Guide to Sugarcane Disease (pp 38–44) La librairie du Cirad, Montpellier Franc.
35. Royer M, Costet L, Vivien E, Bes M, Cousin A, Damais A, Pieretti I, Savin A, Megessier S, Viard M, Frutos R, Gabriel DW and Rott P (2004) Albicidin pathotoxin produced by *Xanthomonas albilineans* is encoded by three large PKS and NRPS genes present in a gene cluster also containing several putative modifying, regulatory, and resistance genes. Mol. Plant-Microbe Interact. 17:414-427.
36. Saumtally S, Medan H and Autrey LJC (1996) Evolution of aerial infection of leaf scald caused by *Xanthomonas albilineans* (Ashby) Dowson in sugarcane. Proceedings International Society of Sugar Cane Technologists 22: 493–496.
37. Schenck S and Lehrer AT (2000) Factors affecting the transmission of *Sugarcane yellow leaf virus*. Plant Dis., 84: 1085-1088.
38. Schenck S (2001) Sugarcane yellow leaf syndrome: history and current concepts. In: Sugarcane pathology. Vol. II: Virus and Phytoplasma Diseases. G.P. Rao, R.E. Ford, M. Tosic and D.S. Teakle (Eds). Science Publishers Inc, Enfield, USA pp. 25-35.
39. Tai PYP, Miller JD and Dean JL (1981) Inheritance of resistance to rust in the sugarcane field. Crops Res 4:261–268.
40. Viator BJ, Griffin JL and Ellis JM (2002) Red morningglory (*Ipomoea coccinea*) control with sulfentrazone and azafeniden applied at layby in sugarcane (*Saccharum spp.*). Weed Technol. 16:142-148.
41. Walker DIT (1971) Breeding for resistance. In: Heinz JD (ed) Sugarcane Improvement Through Breeding (pp 445–502) Elsevier Science Publishers B.V., Amsterdam.

Chapitre 3 : Travaux de recherche, perspectives

3.1 L'échaudure des feuilles :

Xanthomonas albilineans est l'agent causal de l'échaudure des feuilles, maladie présente en Guadeloupe et dans la majorité des aires de culture de cette plante. Les seuls moyens de lutte sont préventifs. Ils impliquent une protection des frontières, des techniques de multiplication et de distribution des plants parfaitement contrôlées ainsi qu'une sélection sévère des nouvelles variétés conduisant à la plantation de variétés résistantes. La durabilité de la résistance est très souvent limitée par la variabilité de l'agent pathogène et son évolution. La connaissance de cette variabilité ainsi que des facteurs génétiques favorisant la pathogénie est essentielle pour une sélection efficace de variétés résistantes à long terme.

Variabilité de l'agent causal de l'échaudure des feuilles

La séquence complète du génome de cette bactérie, prochainement disponible, devrait permettre d'identifier les gènes responsables de la pathogénie de *X. albilineans*. La variabilité et l'évolution de ces gènes pourront alors être étudiées in situ.

Epidémiologie :

Comme beaucoup de bactéries phytopathogènes notamment du genre *Xanthomonas*, certaines souches de *X. albilineans* possèdent une aptitude pour la colonisation épiphyte des feuilles de canne à sucre. Cette colonisation est probablement favorisée par les phénomènes d'ingression égression des bactéries au niveau de la feuille notamment au niveau des hydathodes et des stomates. La survie épiphyte de ces bactéries nécessite aussi la présence épisodique d'eau à la surface des feuilles favorisant ainsi leur déplacement. Cette phase épiphyte est un élément clé dans le cycle infectieux de la bactérie.

Si l'importance du climat sur la dynamique des populations épiphytes a été appréhendée ces dernières années (résultats non publiés, en cours de rédaction) en revanche, l'impact de la résistance des variétés cultivées sur les populations épiphytes de *X. albilineans* n'est pas encore clairement défini et nécessite un complément d'étude. Y-a-t-il un lien entre résistance variétale et dynamique des populations à la surface des feuilles ? Les plantes peuvent-elles lutter contre le développement de ces populations épiphytes ? La réponse à ces questions est primordiale pour améliorer les méthodes de lutte contre l'échaudure des feuilles.

3.2. La maladie de la feuille jaune

La maladie de la feuille jaune est aujourd'hui largement distribuée dans le monde et est associée à un *polerovirus* de la famille des *Luteoviridae* (*Sugarcane yellow leaf virus* ou SCYLV). Propagé de plante à plante par des insectes vecteurs (pucerons) le virus peut infecter les pépinières de canne à sucre issues de matériel végétal sain. Par la suite, le SCYLV peut se propager lors de plantations effectuées avec des boutures issues de tiges infectées. Toutefois, le rôle des pucerons vecteurs dans la propagation du virus peut varier suivant les zones géographiques. Caractériser cette maladie et notamment l'interaction plante hôte vecteur dans les conditions tropicales de Guadeloupe et Martinique est primordial afin d'identifier les facteurs limitant les pertes que pourrait engendrer une épidémie du SCYLV aux Antilles françaises.

Environnement et épidémiologie :

Le SCYLV, dont plusieurs souches virales sont bien implantées en Guadeloupe et Martinique, est véhiculé par *Melanaphis sacchari* qui est responsable des contaminations de matériel végétal sain en Guadeloupe. Ces contaminations font déjà l'objet d'études approfondies afin de modéliser les épidémies à l'échelle de la parcelle de canne à sucre. En revanche, aucune information n'est disponible quant à la dynamique du vecteur *M. sacchari*, vecteur principal du virus en Guadeloupe, en fonction des différentes zones climatiques et des variétés en présence. Relier ces informations à une étude de répartition de la maladie nous permettra de clarifier les spécificités de la dissémination du virus dans les conditions tropicales insulaires de Guadeloupe et Martinique. Ces études seront réalisées en collaboration avec l'UAG.

Etude des relations plante/vecteur/virus :

Le mode de transmission du SCYLV par les pucerons s'effectue selon un mode circulant persistant. De ce fait, la diversité du virus est limitée par la forte spécificité structurelle liée aux barrières à franchir lors de la circulation du virus dans l'insecte vecteur. Malgré cette particularité, plusieurs souches pourraient être transmises par l'insecte et infecter la plante. Est-ce que certaines souches sont mieux adaptées à cette interrelation vecteur/plante, existe-t-il une relation vecteur/souche ou plante/souche ? C'est à ces questions que nous nous proposons de répondre en collaboration avec l'UMR BGPI de Montpellier.

Si des variétés résistantes ont pu être identifiées, les mécanismes mis en jeu sont inconnus. La plante peut en effet avoir des effets antixénose ou antibiose sur l'insecte, ou bien bloquer la transmission du virus par l'insecte ou encore limiter, stopper, la circulation du virus dans le système vasculaire. Déterminer les stratégies de résistance développées par la plante hôte est non seulement nécessaire à la création et au développement de variétés résistantes mais pourrait aussi aboutir à identifier de nouveaux mécanismes de résistance.

Chapitre 3 : Publications sélectionnées

Purification and characterization of two basic Beta-1,3-glucanases induced in *Colletotrichum lindemuthianum* infected bean seedlings . p 29

A putative major gene for rust resistance linked with a RFLP marker in sugarcane cultivar R570. p 36

Protoporphyrinogen oxidase inhibitor herbicide effects on pythium root rot of sugarcane, *Pythium* species, and the soil microbial community p 42

Aerial contamination of sugarcane in Guadeloupe by two strains of *Xanthomonas albilineans*. p 49

High variation in pathogenicity of genetically closely related strains of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen, in Guadeloupe p 63

Variation in infection capacity and in virulence exists between genotypes of *Sugarcane yellow leaf virus*. p 74

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 292, No. 2, February 1, pp. 468–474, 1992

Purification and Characterization of Two Basic β-1,3-Glucanases Induced in *Colletotrichum lindemuthianum*-Infected Bean Seedlings

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Received July 12, 1991, and in revised form September 16, 1991

Two β-1,3-glucanases which are rapidly induced in the incompatible interaction between bean (cv. Processor) and *Colletotrichum lindemuthianum* race β were purified to homogeneity. Characterization of the two enzymes, GE1 and GE2, showed that they both had a basic isolectric point and a similar molecular weight (36,500 for GE1 and 36,000 for GE2), but differed in their pH optimum, thermal stability, and specific activity. GE2 was present in higher amounts but was shown to be less active than GE1 against laminarin and fungal cell walls isolated from race β of the fungus. Both enzymes were specific for β-1,3 linkages and showed a strict endolytic mode of action. Further characterization of GE2 was achieved by amino acid sequence analysis of tryptic peptides; the degree of homology shared with other basic β-1,3-glucanases depended on the plant source. A time-course study showed that GE1 and GE2 were increased during infection. They were also induced by fungal elicitors, thereby indicating that they originate from the host.

Pathogens, elicitors, and diverse stress conditions induce important changes in plant metabolism. Among them, the accumulation of soluble proteins, the so-called pathogenesis-related (PR) proteins, first reported several years ago (1–4), has received much attention (5–9). The recent finding that some of these proteins exhibit chitinase or β-1,3-glucanase activity represents an important step toward understanding their biological significance (5, 10). Although the physiological function of these enzymes is not precisely known, they have the potential to inhibit fungal growth and to hydrolyze fungal cell walls *in vitro* (7). Among the released oligosaccharides, some could act as elicitors of the defense response in plants (11).

Several isoforms of these enzymes, either acidic or basic, are now identified in a few plants. The treatment of bean seedlings with ethylene has been demonstrated to induce β-1,3-glucanase activity, which was mostly due to the appearance of one enzyme which had an isoelectric point of either 11 (12) or 9.7 (8) according to the respective authors. Likewise, basic proteins represent a major part of the extractable β-1,3-glucanase activity found in bean seedlings infected by *Colletotrichum lindemuthianum* (13). The induction of this enzyme has been found to be rapid in the case of an incompatible interaction, whereas it was delayed during a compatible one (13). This difference in the onset of glucanase induction is consistent with a possible role of the enzyme in active plant defense. In order to elucidate further the role of these enzymes, we have purified and characterized two β-1,3-glucanases which appear rapidly in the incompatible interaction between *Phaseolus vulgaris* cv. Processor and *C. lindemuthianum* race β.

MATERIALS AND METHODS

Chemicals and reagents. Chemicals for column chromatography and polyacrylamide gel electrophoresis, as well as molecular weight markers, were obtained from Pharmacia (France), Silicagel 60 F254 was purchased from Merck, and enzyme substrates were obtained from Sigma.

Biological material. Seedlings of *P. vulgaris* L. cv. Processor, resistant to race β of *C. lindemuthianum*, were grown in a greenhouse as described (14). The fungus, *C. lindemuthianum* race β, was maintained on glucose–peptone agar (15). The conidia obtained from a 6-day-old culture of the fungus were suspended in sterile water and used for plant inoculation as described previously (14).

Fungal cell wall and elicitor preparations. Cell walls of *C. lindemuthianum* race β were isolated as described by Ayers *et al.* (16). An elicitor preparation was obtained by boiling the cell walls in water (120 mg/7.5

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0003-9861/92 $3.00

ml) at 120°C for 20 min. The cleared supernatant recovered after centrifugation (12,500*g*, 10 min) represented the elicitor fraction.

Elicitor bioassay. Seven-day-old bean seedlings were cut 2 cm below the cotyledons and allowed to absorb 0.024 mg elicitor diluted in 0.3 ml water plus streptomycin (0.1 mg/ml) through the cut section of the petiole in a small test tube. Absorption of the elicitor was completed after 4 to 6 h at 22°C in a growth chamber under light. The cuttings were then allowed to absorb water plus streptomycin (0.1 mg/ml) for an additional 24 or 48 h at 22°C with a 16-h day length. Untreated controls received only water plus streptomycin under the same conditions.

Enzyme extraction and purification. All steps were carried out at 4°C. The leaves (50 g) of 10-day-old seedlings (3 days after inoculation) were used as an enzyme source. They were ground in a mortar with sand (5 g) and suspended in 500 ml of 0.05 M acetate buffer, pH 5.2, containing 1 M NaCl and Polyclar AT (5 g). The homogenate was cleared by centrifugation for 20 min at 15,000*g*. Solid ammonium sulfate was added to the supernatant to 15% saturation, and the mixture was stirred for 1 h before being centrifuged for 15 min at 15,000*g*. The pellet was discarded and solid ammonium sulfate was added to the supernatant up to 85% saturation. After centrifugation (15 min at 15,000*g*) the resulting precipitate was dissolved in 25 ml of 0.02 M acetate buffer, pH 5.2, and dialyzed overnight against 5 liters of the same buffer. The dialysate was cleared by centrifugation (10 min at 4000*g*) and applied to a cationic S–Sepharose fast-flow column (1.2 × 15 cm) equilibrated with 0.02 M acetate buffer, pH 5.2. The column was then washed with the same buffer and eluted with 400 ml of a linear gradient of NaCl (0 to 0.4 M) in acetate buffer at a flow rate of 50 ml per hour. The active fractions corresponding to two peaks were recovered and pooled separately before being subjected to electrofocusing on a LKB 8000 preparative column (pH 9–11); glycerol was used as the density gradient medium. The anode was at the top of the column and the separation was performed at 600 V for 48 h. The active fractions were pooled and desalted on a prepacked PD-10 Sephadex G25 column with 0.02 M acetate buffer, pH 5.2, before being applied to a S–Sepharose fast-flow column (1.5 × 3 cm) equilibrated with 0.01 M acetate buffer, pH 5.2. After washing the column with the same buffer, enzyme elution was performed with 100 ml of a linear gradient (0.01–0.1 M) of citrate-phosphate buffer at pH 7.0. The active fractions were desalted on a prepacked PD-10 Sephadex G25 column equilibrated and eluted with distilled water, and then lyophilized.

Enzyme assay. β-1,3-Glucanase activity was determined by measuring the amount of reducing sugar released from laminarin. The standard assay (1 ml) contained enzyme extract, 0.2 M acetate buffer (pH 5.2), 2.5 mg laminarin, and 5 μg BSA.[3] After incubation at 50°C (30 min) the reducing sugar content was determined according to the method of Somogyi (17). Appropriate controls, where either the enzyme or the substrate was omitted, were run simultaneously. One nanokatal (1 nkat) corresponds to the release of 1 nmol glucose equivalent $\cdot s^{-1}$ under the above conditions.

Analysis of β-1,3-glucanase reaction products. Substrate hydrolysis by the purified bean β-1,3-glucanases was performed by incubating the enzyme (50 nkat) with 50 mg of laminarin (a β-1,3-glucan) in 10 ml of 0.02 M acetate buffer, pH 5.2, containing BSA (100 μg) at 50°C. Samples (1 ml) were withdrawn at different time intervals and the reaction was stopped by adding 2 ml of ice-cold acetone and keeping the mixture at −20°C. Insoluble material was precipitated by centrifugation, and hydrolysis products present in the supernatant, as well as the standard sugars, were chromatographed on 0.2-mm-thick silica gel 60 F254 plates. The plates were developed three times in acetonitrile:water (4:1, v/v) as described by Mauch *et al.* (7). The sugars were visualized by spraying the chromatogram with a 0.5% (w/v) thymol solution in ethanol containing 5% (w/v) sulfuric acid, followed by heating for 15 min at 110°C.

Hydrolysis of fungal cell walls. Enzymic degradation by β-1,3-glucanases of the isolated cell walls was performed in 6 ml of 0.2 mM sodium acetate buffer (pH 5.2) containing 120 mg isolated cell walls and enzyme solution (5 nkat). After incubation of the mixture at 50°C for various times under constant stirring, the reaction was stopped by centrifugation at 12,500*g*. The amount of reducing sugars released in the supernatant was determined by the method of Somogyi (17).

Gel electrophoresis. Native gel electrophoresis under acidic conditions was performed according to Reisfeld *et al.* (18) except that Temed and ammonium persulfate concentrations were 0.8 and 0.15%, respectively, and the reservoir buffer consisted of 80 mM alanine and 40 mM acetic acid (pH 4.4). The stacking and separating gels were 3 and 10% in acrylamide, respectively. Electrophoresis under denaturing conditions (SDS–PAGE) was performed by the method of Laemmli (19) with 3% acrylamide in the stacking gel and 12.5% acrylamide in the separating gel. Gels were stained with silver nitrate according to Morrissey (20). Western blots were incubated with an antiserum against tobacco β-1,3-glucanase and immunological detection of the antigen–antibody complexes was carried out as described by Benhamou *et al.* (21).

Protein determination. The protein content of column eluates was monitored by measuring the absorption at 280 nm. Quantitative determinations were performed with bicinchoninic acid according the manufacturer (Pierce). For each measurement, a standard plot was established with BSA (Sigma).

Amino acid sequence analysis. Purified GE2 β-1,3-glucanase was digested with trypsin for 15 h at 20°C; the protein/trypsin ratio was 60/1 (w/w). The resulting peptides were separated on a C18 reversed-phase HPLC column with a 0 to 70% (v/v) linear gradient of acetonitrile in H_2O containing 0.1% TFA. Amino acid sequence analysis of the recovered peptides was performed with an Applied Biosystems 470 A/120 A protein sequencer according to the manufacturer. Tryptic digestion and sequencing of the GE2 peptides were performed by Sanofi Elf Biorecherches-Labège Innopole, France.

RESULTS

The protein extracts obtained by ammonium sulfate fractionation of the homogenates prepared from 10-day infected bean seedlings and uninoculated controls were first purified by cation-exchange chromatography on a S–Sepharose column. Three peaks of β-1,3-glucanase activity were recovered from extracts of inoculated resistant seedlings (Fig. 1). The peak which was not retained appeared to be a constitutive β-1,3-glucanase since it was also present in healthy controls; in contrast, the additional two peaks, called GE1 and GE2, were specifically induced by inoculation with *C. lindemuthianum*. Due to their cationic nature they differed from the acidic enzyme present in the fungus (13). GE1 and GE2 were further purified by two additional steps. Electrofocusing and a second cation-exchange chromatography step allowed us to noticeably increase the specific activity of GE1 and GE2 (Table I). GE1 was less abundant, but more active, than GE2. The proteins present in the active fractions at various stages of the purification were analyzed by electrophoresis. The two enzymes recovered after the last ion-exchange chromatography step appeared to be homogeneous since, over a range of increasing concentrations, only one band was observed in native gels (Figs. 2a and 2b) at an acidic pH (pH 4.4) as well as under denaturing

[3] Abbreviations used: BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; IWF, intercellular washing fluids; PR, pathogenesis-related.

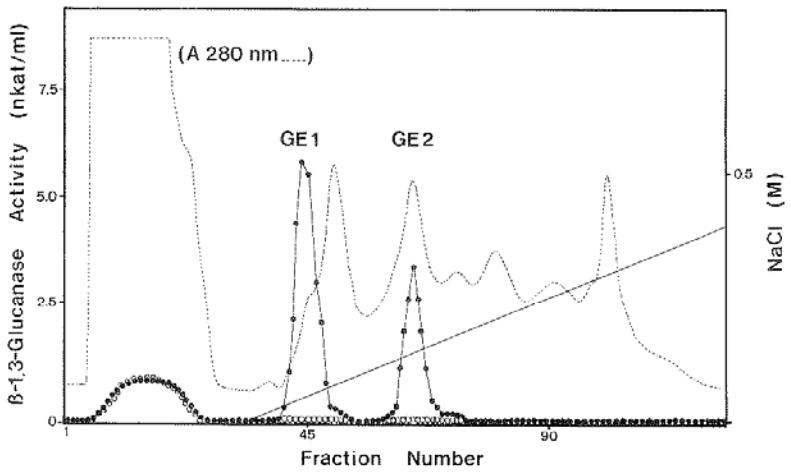

FIG. 1. Chromatography on a S–Sepharose fast-flow column of the extract obtained from pathogen-challenged (●) and uninoculated (○) bean seedlings after ammonium sulfate fractionation. Elution of the column was performed at a flow rate of 50 ml per hour, as described under Materials and Methods; the fraction volume was 5 ml.

conditions in the presence of SDS (Figs. 2c and 2d). They differed from each other by their electric charge, GE2 being more basic than GE1.

The physicochemical parameters which were determined are listed in Table II. The molecular weights of GE1 and GE2 were 36,500 and 36,000, respectively, as calculated from their migration during SDS–PAGE. Other calibration methods using gel filtration could not be employed in this case because the two enzymes had an affinity for the Sephacryl S-200 and Sephadex G-100 gels suitable for this analysis and hence would be retained on the column. The isoelectric points of the two induced enzymes were deduced from their migration upon electrofocusing. The values of 10.1 and 10.5 were obtained for GE1 and GE2, respectively, thereby indicating their basic nature.

Both glucanases were active over a broad range of pH values (2.5 to 6.5), GE1 being maximally active in citrate-phosphate buffer at pH 5.8 and GE2 at pH 5.0. The effect of temperature was determined by carrying out the enzyme assays between 25 and 70°C for 30 min. The optimum temperatures for laminarin hydrolysis were 50 and 45°C, respectively, for GE1 and GE2. GE1 had a low thermal stability and completely lost its activity after 1 h at 50°C or 2 h at 30°C. In contrast, GE2 was much more stable, and lost only 30% of its activity after 1 h at 50°C.

Measurement of the activities of GE1 and GE2 against a wide range of disaccharides and polysaccharides showed that the two enzymes were specific for β-1,3-linked glucans (laminarin). They did not hydrolyze pustulan (β-1,6), xy-

TABLE I

Purification of Two β-1,3-Glucanases from Inoculated Bean Seedlings

Purification steps		Protein (mg)	Activity (nkat)	Specific activity (nkat/mg protein)	Recovery (%)
Crude extract		384	572	1.5	100
Ammonium sulfate precipitation		77.1	420	5.4	73
S–Sepharose chromatography					
(pH 5.2)	GE1	2.6	122	46.9	21.3
	GE2	6.8	61	9.0	10.6
Electrofocusing	GE1	0.45	60	131	10.5
	GE2	0.80	21.2	26.2	3.7
S–Sepharose chromatography					
(pH 7.0)	GE1	0.073	44	603	7.7
	GE2	0.296	16.9	57.1	2.2

Note. The starting material (50 g fresh weight) consisted of 10-day-old bean seedlings inoculated with *C. lindemuthianum* race β for 72 h.

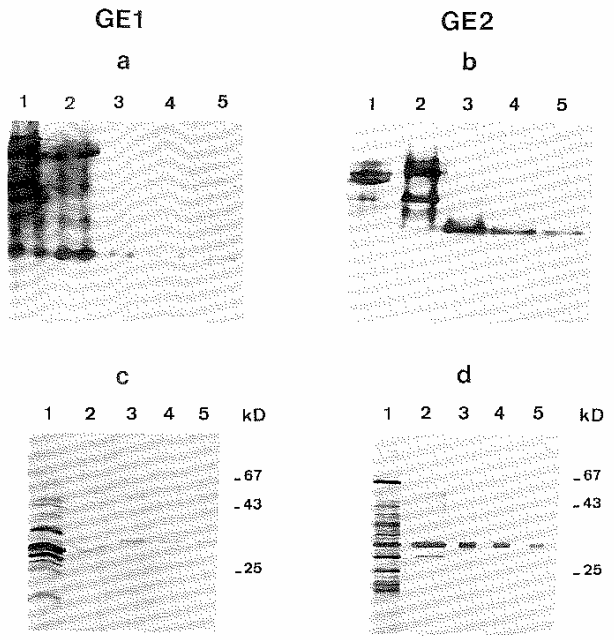

FIG. 2. Polyacrylamide gel electrophoresis of GE1 and GE2 under native or denaturing conditions. (a and b) Native gel electrophoresis was performed in a 10% polyacrylamide gel at pH 4.4; lanes 1 and 2, protein extract (6 μg) corresponding to the active fractions recovered after S–Sepharose chromatography (pH 5.2) and electrofocusing, respectively; lanes 3–5, samples containing 0.2, 0.1, and 0.05 μg of GE1 (a) or GE2 (b) respectively, recovered after the last S–Sepharose chromatography step (pH 7.0). (c and d) SDS–PAGE was performed in a 12.5% polyacrylamide gel with the same protein extracts (lanes 1 and 2) and GE1 (c) or GE2 (d) samples (lanes 3–5) as above. Standard molecular weight proteins (bovine albumin, M_r 67,000; ovalbumin, M_r 43,000; chymotrypsinogen A, M_r 25,000; and ribonuclease, M_r 13,500) were run simultaneously for calibration.

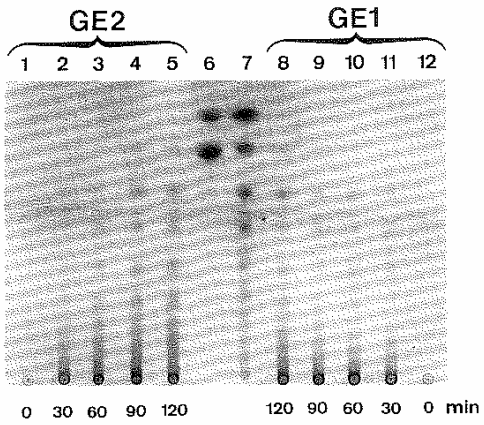

FIG. 3. Thin-layer chromatography of the products recovered from laminarin upon enzymic hydrolysis with GE2 (lanes 1–5) and GE1 (lanes 8–12). Lanes 1 and 12, unhydrolyzed samples; lanes 2–5 and 8–11, samples recovered after 30, 60, 90, and 120 min of incubation; lane 6, a standard mixture of glucose and laminaribiose (10 μg); and lane 7, products recovered from laminarin upon hydrolysis by 1 M TFA for 1 h at 80°C.

lan (β-1,4), carboxymethylcellulose (β-1,4), nigeran (α-1,3 and α-1,4), and chitin (β-1,4). They were both inactive against disaccharides such as laminaribiose (β-1,3), gentiobiose (β-1,6), and cellobiose (β-1,4). Accordingly, both induced enzymes are β-1,3-glucanases. In order to better define whether they acted as endo- or exoglucanases, the products released from laminarin were analyzed by thin-layer chromatography (Fig. 3). The products obtained after 30 min hydrolysis at 50°C ranged from the disaccharide laminaribiose to longer oligosaccharides. It was deduced from this experiment that GE1 and GE2 are true endo-β-1,3-glucanases. Of the two enzymes, GE1 was the most active against laminarin.

GE1 and GE2 were also assayed for *in vitro* digestion of the cell walls isolated from *C. lindemuthianum* race β. Both enzymes released soluble oligosaccharides from the cell walls (Fig. 4). Again, GE1 was at least twice as active

TABLE II

Physicochemical Characterization of the Two Enzymes GE1 and GE2 Induced in Processor-Resistant Bean Seedlings upon Inoculation with *C. lindemuthianum* Race β

Chemical parameters	GE1	GE2
M_r	36,500	36,000
p*I*	10.1	10.5
Optimum pH	5.8	5.0
Activity remaining		
After 1 h at 30°C	35%	100%
After 1 h at 50°C	0%	70%
Substrate specificity	β-1,3-glucan	β-1,3-glucan

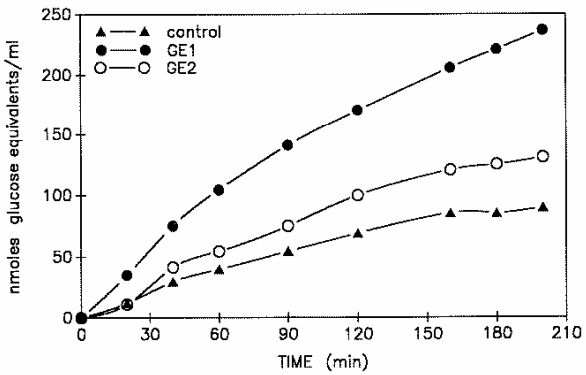

FIG. 4. Time-course of the release of soluble reducing oligosaccharides from *C. lindemuthianum* cell walls by GE1 (●) and GE2 (○). The control (▲) corresponds to the incubation of a cell wall preparation alone in the same conditions. Values are the means of three different experiments.

	55 64	111 125	180 193
GE2	LYDPNQAALQALR	YIAVGN*VSPVGGSE	YLDPII*YL*YASA
Bean	.....G...G...	......E.......S	......G..L....
Tobacco	.....HG.....K	......EI...T.TS	FT...VGF.RDTR.
Soybean	I.G.SPEV.E...	.VS...E.K.EHSFA	...GV.RF.VNNN.

FIG. 5. Comparison of the sequence of three peptides obtained by trypsin digestion of GE2 with known sequences of basic β-1,3-glucanases from bean cell cultures, tobacco cell suspension culture, and ethylene-treated soybean plants. Sequence alignment was assessed visually. Amino acids identical to those of GE2 are shown as a dot. Numbers indicate positions in the tobacco sequence, and asterisks indicate residues that could not be identified unambiguously.

as GE2. The activity represented approximately 5% of the maximum activity obtained with laminarin; such a difference resulted from the highly complex structure of the cell wall. The release of sugars from control cell walls, i.e., without added enzyme, might reflect the presence of an endogenous glycosidase activity in fungal cell walls.

The identity of the purified enzyme GE2 was further confirmed by analysis of three peptides obtained after trypsin digestion and separation by reversed-phase HPLC. Their amino acid sequences (Fig. 5) were compared with sequences deduced from cDNA clones of basic glucanases prepared from hormone-induced tobacco cell suspension (22), ethylene-treated soybean cotyledons (23), and elicitor-treated bean cell suspension (24). Homology between GE2 and other glucanases was high for the bean glucanase, moderate for tobacco glucanase, and very low for the soybean enzyme. Preliminary data showed that GE1 had a different peptide pattern, thereby suggesting that the two enzymes are different from each other at the protein level. This, however, awaits further confirmation.

Additional experiments were performed in order to study the biological significance of GE1 and GE2. A time-course analysis of race-β-infected bean seedlings showed that GE1 and GE2 appeared as early as 3 days after inoculation in resistant seedlings and were highly increased at Day 5 (Fig. 6a). Simultaneously, the constitutive activity was only slightly increased. In order to look for the accumulation of β-1,3-glucanases at the subcellular level, entire leaves were infiltrated with distilled water *in vacuo* and the intercellular washing fluids were recovered according to Joosten and De Wit (9). Malate dehydrogenase activity, which served as a control cytoplasmic enzyme, was not detected in the extracts. This procedure allowed us to show that at least part of the total β-1,3-glucanase activity was present in the intercellular spaces and that the activity starts increasing 2 to 3 days after inoculation (Fig. 7). SDS–polyacrylamide gel electrophoresis of the intercellular fluids showed that, among additional protein bands appearing upon infection (Fig. 8a, lane 2), one of them had the same electrophoretic mobility as the major protein present in leaf extracts containing GE1 and GE2 (lane 3). In a separate experiment, GE1, GE2, and the intercellular washing fluids of infected and uninfected leaves were run together and then blotted and probed with an antiserum against the acidic PR-O tobacco β-1,3-glucanase (kindly provided by Dr. B. Fritig). The data

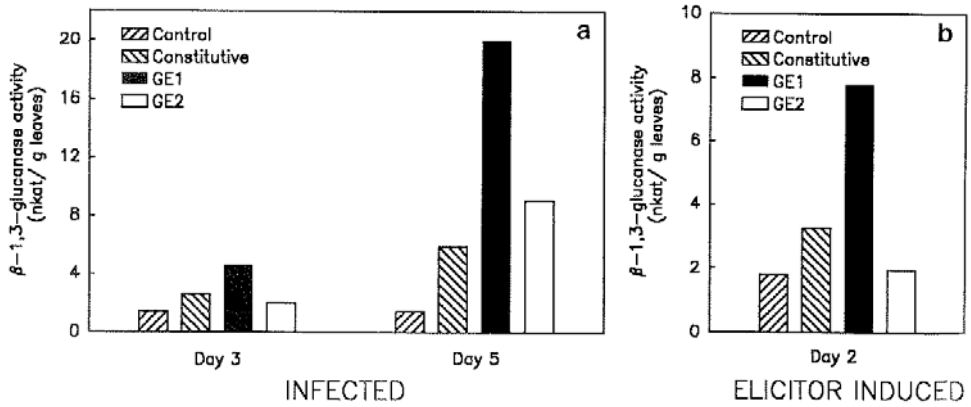

FIG. 6. Time-course measurement of GE1, GE2, and constitutive β-1,3-glucanases during infection (a) and elicitor treatment (b) of resistant bean seedlings. The proportion of constitutive, GE1, and GE2 glucanase activity was monitored after separation by cation-exchange chromatography of the leaf extracts prepared from 3- or 5-day infected plants (a) and from 48-h elicitor-treated seedlings (b). Control represents the total β-1,3-glucanase activity from uninoculated bean leaves or unelicited cell cultures.

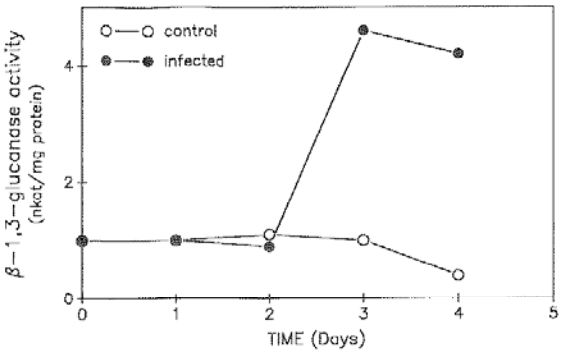

FIG. 7. Time-course analysis of β-1,3-glucanase activity in the intercellular washing fluids of inoculated resistant bean leaves (●) and uninoculated controls (○). SDS–PAGE of the corresponding protein extracts is shown in Fig. 5.

(Fig. 8b) showed that the two glucanases reacted with the antiserum (lanes 4 and 5) and that only one form was present in the intercellular washing fluids of infected leaves (lane 6). This form apparently migrated like GE2 and was absent in the intercellular fluids of control plants (lane 7).

Early changes in β-1,3-glucanase activity were also recorded in response to heat-released elicitors from the fungus (Fig. 6b). Maximum increase was reached 24 h after beginning the treatment. The β-1,3-glucanase activity of the protein extract, prepared from seedlings treated for 48 h and purified by cation-exchange chromatography, was composed of 25% constitutive activity, 60% GE1, and 15% GE2, thus showing proportions similar to those in extract from infected plants. This experiment also demonstrated that GE1 and GE2 originate from the host and not from the pathogen.

DISCUSSION

Pathogenesis-related proteins are induced in bean seedlings in response to viruses, ethylene, and mercuric chloride (4, 7, 8, 25). In a preceding paper, we have reported that β-1,3-glucanase activity increases early during the incompatible interaction between bean seedlings and *C. lindemuthianum* (13). The present work is the first report that two basic enzymes, called GE1 and GE2, account for the β-1,3-glucanase activity which is induced in bean upon fungal infection.

GE1 and GE2 were purified to provide material for characterization studies. The two enzymes, which were recovered after several chromatography steps and electrofocusing, appear homogeneous upon gel electrophoresis. They account for a major proportion (40%) of the total β-1,3-glucanase activity which is detected in inoculated seedlings.

Several lines of evidence suggested that GE1 and GE2 are distinct isoforms rather than one being a degradation product of the other: (a) they were clearly distinguishable from each other on the basis of their physicochemical parameters (optimum catalytic pH, thermal stability, specific activity, p*I*, and migration upon native gel electrophoresis); (b) heterogeneity was not observed upon chromatography or electrophoresis under various conditions, even when loading a very high protein concentration; and (c) they appear every time in response to elicitors as well as to infection. Their M_r, 36,500 (GE1) and 36,000 (GE2), respectively, are similar to those of other basic β-1,3-glucanases isolated from several plants (5–8, 25, 26). These values fall within the low M_r range reported for all β-1,3-glucanases identified so far, whether constitutive or inducible, acidic or basic. In partially purified extracts (Fig. 5, lane 3), they migrate as two distinct chemical entities. The occurrence of two closely related basic β-1,3-glucanase isoforms has already been described in other plant–fungi interactions (6, 7, 9). For example, in *Phytophthora infestans*-infected potatoes, M_r of 36 and 36.2 kDa were reported for the two isoforms (6).

GE1 was not a glycosylated isoform of GE2, since sugar residues could not be detected in their hydrolysates by conventional techniques, nor did they stain for glycoproteins after electrophoresis. Whether the two β-1,3-glucanases are encoded by one gene or by two different genes is not known. A preliminary experiment showed a quite different tryptic peptide pattern between GE1 and GE2, suggesting that they are encoded by two different genes. Induction of the two forms by elicitor treatment demonstrated that they are encoded by the host and not by the pathogen. The biochemical data available on the ethylene-induced basic enzyme in bean seedlings (7, 12, 25, 27) indicate that this enzyme shares features in common

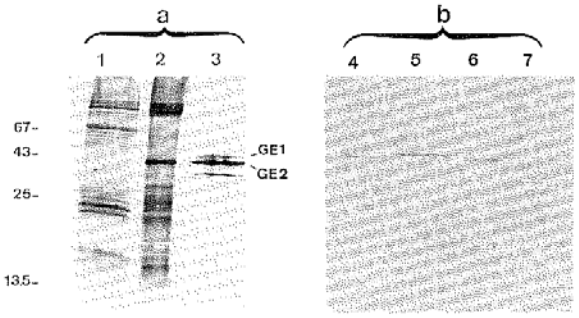

FIG. 8. SDS–PAGE analysis of proteins in the intercellular washing fluids (IWF) and in cellular extract of resistant bean leaves (a), and immunoblot analysis of the IWF and of the purified β-1,3-glucanases (b). (a) IWF from uninfected (lane 1) and infected (lane 2) bean leaves, 3 days after inoculation. Lane 3 corresponds to 3 μg of GE1 and GE2 recovered from leaf extracts after electrofocusing. (b) 0.25 μg of GE1 (lane 5) and GE2 (lane 4), and the IWF from infected (lane 6) and uninfected (lane 7) leaves were transferred to nitrocellulose and probed with an antiserum raised against tobacco β-1,3-glucanase at a 1:1000 dilution.

with infection-induced GE1 and GE2 enzymes (such as M_r and p*I*) but are not sufficient to clearly assign identity of this protein to either form induced by infection.

Recently, a cDNA clone of bean β-1,3-glucanase has been isolated (24). Amino acid sequence comparison of three GE2 tryptic peptides with the amino acid sequences deduced from this clone indicated a high degree of homology. Only a partial homology was found with the β-1,3-glucanases from other plant sources (tobacco, soybean). Indeed, the present knowledge of amino acid sequences obtained from proteins or deduced from cDNA clones indicates a rather low degree of homology between acidic and basic β-1,3-glucanases found in one plant as well as between those from different plants.

Although extensive biochemical data are now available on PR proteins, notably β,1-3-glucanases, only a few studies have thoroughly elucidated the question of substrate specificity (11, 12, 28, 29). The two enzymes purified in this work were specific for β,1-3 linkages and exhibit an endolytic activity similar to that of β-1,3-glucanases from other plant sources such as rye, soybean, and tobacco (11, 28, 29). β-1,3-Glucans are found both in fungal cell walls and in callose deposits in plants. In this work, we demonstrate that GE1, and to a lower extent GE2, is able to hydrolyze *C. lindemuthianum* cell walls *in vitro*. Hydrolysis of the cell wall β-1,3-glucans might account for the defense of plants against fungi and for the release of elicitor-active molecules which contain β-1,3 linkages. Indeed, an endolytic mode of action would appear suitable for the postulated defense function of these enzymes. The two forms GE1 and GE2 were early induced in resistant bean seedlings in response to infection or to elicitor treatment. The rapid stimulation of β-1,3-glucanase activity parallels the development of the hypersensitive cell necrosis response in this system, thereby suggesting a possible role in the outcome of this reaction.

Ethylene treatment induced one unique basic β-1,3-glucanase in beans (8, 12, 25), whereas infection and elicitors induced two isoforms. In contrast to the ethylene-induced enzyme, which is mainly located in the vacuole (30), we showed that an important part of the infection-induced activity seemed to be present in the intercellular fluids of bean leaves. In potato, the two basic β-1,3-glucanases induced by *P. infestans* or by fungal elicitors were also present in the apoplastic fluid (6). Future studies should allow a better knowledge of bean β-1,3-glucanases at the molecular level.

ACKNOWLEDGMENTS

We are grateful to Dr. B. Fritig (IBMP–CNRS Strasbourg) for kindly providing antiserum against PR-O β-1,3-glucanase and to Dr. Martina Rickauer for helpful discussions and contributions during the preparation of the manuscript. Part of this work was supported by Sanofi Elf Biorecherches-Labège Innopole, France.

REFERENCES

1. Gianinazzi, S., Pratt, H. M., Shewry, P. R., and Miflin, B. J. (1977) *J. Gen. Virol.* **34**, 345–351.
2. Van Loon, L. C., and Van Kammen, A. (1970) *Virology* **40**, 199–211.
3. Van Loon, L. C. (1985) *Plant Mol. Biol.* **4**, 111–116.
4. De Tapia, M., Bergmann, P., Awade, A., and Burkard, G. (1986) *Plant Sci.* **45**, 167–177.
5. Kauffmann, S., Legrand, M., Geoffroy, P., and Fritig, B. (1987) *EMBO J.* **6**, 3209–3212.
6. Kombrinck, E., Schröder, M., and Hahlbrock, K. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 782–786.
7. Mauch, F., Hadwiger, L. A., and Boller, T. (1988). *Plant Physiol.* **87**, 325–333.
8. Vogeli, U., Meins, F., and Boller, T. (1988) *Planta* **174**, 364–372.
9. Joosten, M. H. A. J., and De Wit, P. J. G. M. (1989) *Plant Physiol.* **89**, 945–951.
10. Legrand, M., Kauffmann, S., Geoffroy, P., and Fritig, B. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 6750–6754.
11. Keen, N. T., and Yoshikawa, M. (1983). *Plant Physiol.* **71**, 460–465.
12. Abeles, F. B., Bosshart, R. P., Forrence, L. E., and Habig, W. H. (1971) *Plant Physiol.* **47**, 129–134.
13. Daugrois, J. H., Lafitte, C., Barthe, J. P., and Touzé, A. (1990) *J. Phytopathol.* **130**, 225–234.
14. Lafitte, C., Barthe, J. P., Montillet, J. L., and Touzé, A. (1984). *Physiol. Plant Pathol.* **25**, 39–53.
15. Bannerot, H. (1965) *Ann. Amelior. Plant.* **15**, 201–222.
16. Ayers, A. R., Ebel, J., Valent, B., and Albersheim, P. (1986) *Plant Physiol.* **57**, 760–765.
17. Somogyi, M. (1952) *J. Biol. Chem.* **195**, 19–23.
18. Reisfeld, R. A., Lewis, V. J., and Williams, D. J. (1962) *Nature (London)* **195**, 156–160.
19. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
20. Morrissey, J. H. (1981) *Anal. Biol. Chem.* **117**, 307–310.
21. Benhamou, N., Parent, J. G., Garzow, S., Asselin, A., Ouellett, G. B., and Jaly, J. R. (1987). *Can. J. Plant Pathol.* **9**, 106–114.
22. Shinshi, H., Wenzler, H., Neuhaus, J. M., Felix, G., Hofsteenge, J., and Meins, F. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5541–5545.
23. Takeuchi, Y., Yoshikawa, M., Takeba, G., Tanaka, K., Shibata, D., and Horino, O. (1990) *Plant Physiol.* **93**, 673–682.
24. Edington, B. V., Lamb, C. J., and Dixon, R. A. (1991) *Plant Mol. Biol.* **16**, 81–94.
25. Vogeli-Lange, R., Hansen-Geho, A., Boller, T., and Meins, F. (1988) *Plant Sci.* **54**, 171–176.
26. Nasser, W., de Tapia, M., Kauffmann, S., Montasser-Koushari, S., and Burkard, G., (1988) *Plant Mol. Biol.* **11**, 529–538.
27. Abeles, F. B., and Forrence, L. E. (1970) *Plant Physiol.* **45**, 395–400.
28. Ballance, G. M., and Manners, D. J. (1978) *Phytochemistry* **17**, 1539–1545.
29. Felix, G., and Meins, F. (1986) *Planta* **167**, 206–211.
30. Mauch, F., and Staehelin, L. A. (1989) *Plant Cell* **1**, 447–457.

Theor Appl Genet (1996) 92:1059–1064

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A putative major gene for rust resistance linked with a RFLP marker in sugarcane cultivar 'R570'

Received: 2 November 1995 / Accepted: 11 November 1995

Abstract Inheritance of resistance to rust was investigated in the self progeny of the sugarcane cultivar 'R570' also used to build a RFLP genetic map. Resistance was evaluated through both field and controlled greenhouse trials. A clear-cut 3 (resistant) : 1 (susceptible) segregation indicative of a probable dominant resistant gene was observed. This is the first documented report of a monogenic inheritance for disease resistance in sugarcane. This gene was found linked at 10 cM with an RFLP marker revealed by probe *CDSR29*. Other minor factors involved in the resistance were also detected.

Key words Major gene · Polyploidy · *Puccinia melanocephala* · Sugarcane · RFLP · Rust

Introduction

The genome organisation of current sugarcane cultivars is very complex. They are highly polyploid and aneuploid clones derived from interspecific hybridisation between *S. officinarum* L. (2n = 80), the first domesticated species, and a wild relative, namely *S. spontaneum* L. (2n = 40–128). Their chromosome number is in the range of 100 to 130, with a probable 10% contribution from the wild species. The basic chromosome numbers of *S. officinarum* and *S. spontaneum* are most likely different, $x = 10$ and $x = 8$, respectively (Sreenivasan et al. 1987; D'Hont et al. 1995; D'Hont et al. 1996). This genetic complexity makes breeding for all traits including resistance to diseases a difficult task, as qualitative Mendelian trait segregation seems to be the exception. A few morphological characters have been found to be controlled by one or two genes. For ring color, a segregation reported in *S. officinarum* fitted a monogenic determinism in the F_2 but not in the backcross progenies (Raghavan and Govindaswamy 1956). For ligular process, segregation fitted a digenic inheritance in a *S. officinarum* L. × *S. spontaneum* L. cross (Batcha and Palanichamy 1978). For diseases, however, no major resistance gene has yet been documented. The lagging behind of Mendelian genetics in sugarcane is partly due to its genome complexity, especially the high ploidy level of clones, which hides possible existing mutations, and also to practical limitations such as a difficulty in efficiently controlling pollination during crosses.

Common rust of sugarcane is caused by the fungus *Puccinia melanocephala* Syd P. Syd. The disease occurs world-wide and can cause high sugar tonnage losses in susceptible varieties (Purdy et al. 1983; Taylor et al. 1986; Comstock et al. 1992). Rust resistance is generally considered to be quantitatively inherited trait with a high heritability and a strong additive genetic variance component (Tai et al. 1981; Hogarth et al. 1983, 1993). The existence of rust pathogenic races has been reported in India, where the disease is probably endemic (Srinivasan and Muthaiyan 1965), but also in Florida where the disease was first detected in 1979 (Dean and Purdy 1984). In Australia, where rust was first reported in 1978, no pathogenic races have been detected (Taylor 1992).

Molecular markers provide a powerful tool to unravel the complex genome of sugarcane and enhance the determination of Mendelian bases for trait inheritance. In recent years, the use of molecular markers in this crop has increased rapidly. Genetic mapping was first performed on the wild species *S. spontaneum* (A1-Janabi et al. 1993; Da Silva et al. 1995). It was also undertaken on cultivated materials (D'Hont et al. 1994). Recently, a large-scale restriction fragment length polymorphism (RFLP) map was constructed based on a self progeny of cv 'R570' (Grivet et al. 1996).

Communicated by H. F. Linskens

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In the work reported here, we studied rust resistance inheritance in the self progeny of cv 'R570', through both field trials with natural infection and controlled greenhouse trials with artificial inoculation. We identified a discrete 3:1 segregation, which can be attributed to a major dominant resistance gene. This gene was shown to be linked, at 10 cM, with a simple molecular marker revealed by an RFLP probe.

Materials and methods

Plant material

Resistance was evaluated on the self progeny (P1) of the rust-resistant cultivar 'R570' that was used to build an RFLP genetic map (Grivet et al. 1996). 'R570', developed by the Centre d'Essai de Recherche et de Formation (CERF), is commercially very successful in Réunion and Mauritius. Both of the parents of 'R570', 'R445' and 'H328560', are resistant to the rust disease. Marker segregations enabled us to check that progeny individuals were indeed derived from the selfing of 'R570', and this then allowed us to discard a few outstanding individuals. Resistance to rust was evaluated in two different field trials and under controlled conditions in the greenhouse. Due to seed (cuttings) availability, the various trials involved slightly different materials. A second sample of 83 individuals of the self progeny of 'R570', (P2), was used to test the results obtained on the P1 population and improve the accuracy of the conclusions.

Rust susceptibility in the field

Population P1 was evaluated in two field trials planted at the CIRAD breeding station of La Mare (Réunion Island) where the rust pressure is present naturally (Peros and Lombard 1986). The first trial (trial 1) evaluated the susceptibility of 64 clones. It followed a completely randomised layout where each clone was represented by a single plot. Each plot consisted of one 3-m-long row of 6 plants. Rust susceptibility was evaluated on plant cane (as opposed to ratoon crop) in 1992.

The second trial (trial 2) evaluated the susceptibility of 58 clones, with all but 1 already included in trial 1. The clones were planted in a partially (half) balanced lattice layout with three replications. Field plots consisted of two rows 2 m in length with 1.5 m between the rows. Planting density was four three-eye cuttings per meter. Rust susceptibility was evaluated on plant cane in 1993 and first ratoon in 1994.

The P2 population was evaluated for rust susceptibility in 1995, in a randomised complete block layout with three replications. The field plots consisted of 2.5-m long row, with a planting density of four three-eye cuttings per meter.

For all of the trials, the susceptibility level of each plot was graded on a 1 to 9 scale (Tai et al. 1981), 1 being the most resistant (no pustule) and 9 the most susceptible (high pustule density and tissue death).

Rust susceptibility in controlled conditions

Sixty-three clones of P1 were evaluated under controlled conditions through greenhouse trials. The size of the greenhouse limited us to the evaluation of a maximum of 30 clones per trial. We estimated first the repeatability of the test by evaluating the same 20 clones in two different trials. We then evaluated another 43 clones in two other trials. In each trial, 4 clones of known susceptibility (from the most to the least susceptible: 'H49-5', 'B34104', 'R469', 'R570') were used as a control.

The protocol of rust susceptibility evaluation has already been described by Peros (1989). For each clone, 15 single-bud cuttings were planted in 0.8 l soil/pozzolana (v/v) pots. After 12 weeks, the last developed leaf of each of the five largest plants was inoculated by spraying uredospores under a settling tower. The inoculum was taken from field-infected plants and propagated in a greenhouse room on susceptible cultivar 'B4362'. Inoculum was calibrated to give approximately 60 germinated spores per square centimeter on agar medium. Inoculated plants were kept for 16 h at 100% relative humidity and then transferred to a greenhouse room. Disease expression was scored 15 days later by measuring uredinia density on 5 cm^2 per inoculated leaf. For each clone, the susceptibility measure was the mean of density obtained on the five leaves.

Mapping data

The construction of the genetic map on P1 has been reported elsewhere (Grivet et al. 1996). Mapping was performed by using 1 isozyme and 128 RFLP probes. This made 505 simplex markers available for the segregation analysis. Among those, 408 were linked into 96 cosegregation groups. These could be assembled into ten linkage groups on the basis of probes in common. Ninety-six markers remained unlinked. Eight probes had no marker involved in any cosegregation group. By discarding markers with more than 10% missing data, we were able to use 439 markers to search for associations with rust susceptibility. Probe *CDSR29*, which is associated with the main rust effect in P1, was used to reveal a RFLP on P2 according to the same RFLP protocol as Grivet et al. (1996).

Data analysis

The rust susceptibility scores obtained from the greenhouse trial were transformed by square root in order to stabilise the variance. Quantitative data analyses were performed using the SAS computer program (SAS Institute 1988). Normality of the different susceptibility measures was evaluated through the test of Shapiro and Wilk (1965) using the procedure UNIVARIATE. In field trial 2, in which each clone was repeated three times in a partially balanced lattice layout, the least square means were recovered with the GLM procedure. The VARCOMP procedure was used to estimate the genetic variance component σ_g^2 from which the broad-sense heritability on a plot basis, h_1^2 and on an entry mean basis, h_2^2, were deduced:

$$h_1^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2},$$

$$h_2^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{n}},$$

where n is the mean number of replications for each clone, and σ_e^2 the error variance component. The same method was used to estimate the heritabilities of the greenhouse test on the 20 clones evaluated in two different trials, which were considered as two blocks.

Significant associations between each of the 439 markers and the different rust susceptibility measures were detected using the non-parametric test of Kruskal-Wallis available on the computer program MAPQTL 2.2 under development at CPRO-DLO, the Netherlands (J. W. Van Ooijen, personal communication). This test is particularly adapted to treat ordinal data that do not follow a normal distribution (Van Ooijen et al. 1992).

Detection and estimation of linkage between Mendelian factors were performed according to Mather (1957).

Results

Rust susceptibility in the field

Rust susceptibility in population P1 was evaluated on three crops under natural inoculation, two on plant cane (trial 1, 1992· trial 2, 1993) and one on first ratoon (trial 2, 1994). This gave three different evaluations of rust sus-

ceptibility in different ecological (year and trial) and physiological (plant cane and ratoon) environments.

The distributions displayed by the three rust susceptibility measures were all distinct from a normal distribution with the test of Shapiro and Wilk at the 5% level, and tended to be "L shaped" with a majority of resistant clones and a minority of dispersed sensitive clones. All three measurements were highly correlated (Table 1). The heritability of the rust susceptibility was evaluated in the second trial and was very high on an entry mean basis in both plant cane (0.94) and first ratoon (0.97), thereby showing a high genetic variance component and good control of the environmental variation in the trial.

Rust susceptibility under controlled conditions

A set of 20 clones of population P1 were evaluated twice in two different experiments, thereby enabling us to evaluate the heritability of the greenhouse test, which was 0.97 on a plot basis. On the basis of this high value a high level of confidence could be attributed to the estimation of rust susceptibility in a single greenhouse test. We thus evaluated another 43 clones, each clone being repeated once. Variability in the self progeny appeared to be high. While the majority of the clones were as resistant as 'R570', several clones were more susceptible than 'R469', and 1 was as susceptible as 'H49.5'. As for the field trials, the distribution of the greenhouse measurement was not normal and tended to be "L shaped". The greenhouse test was highly correlated with the three field susceptibility measures (Table 1).

Identification of a discrete 3:1 distribution in the progeny

Although rust susceptibility may appear as a quantitatively distributed trait when the different measures are considered independently, a clear-cut segregation was revealed when the two measures were considered simultaneously (Fig. 1). This allowed individual progeny to be classified into two populations, one homogeneous with highly resistant clones and the other heterogeneous, with clones of various susceptibility levels. The clones of this second population will subsequently be designated as "susceptible" for simplification. A discrepancy appeared for 1 clone between field and greenhouse measures. This clone was resistant in the field and very susceptible in the greenhouse. All other clones presented a proportionate response in the greenhouse and the field trials (Fig. 1).

For 63 of the 65 P1 individuals we had a value for at least two of the three measures of susceptibility in the field. It was thus possible to score each of them as "resistant" or "susceptible" on the basis of two-dimensional plots: 45 were quoted "resistant" and 18 "susceptible". The ratio of resistant versus susceptible clones was not different from a 3:1 ratio ($\chi^2 = 0.48$), which is the expected segregation ratio for a simplex dominant resistance gene.

Table 1 Heritability and correlations between the different measures of rust in the field and in controlled conditions on the selfed progeny of R570

Trial	N[a]	h_1^2	h_2^2	Pearson correlation coefficients[b]			
				Trial 1 pc	Trial 2 pc	Trial 2 r	gh trial
Trial 1 pc	64	–	–	–	0.89	0.90	0.79
Trial 2 pc	58	0.86	0.94	–	–	0.95	0.75
Trial 2 r	58	0.93	0.97	–	–	–	0.75
gh trial	63	0.97	0.99	–	–	–	–

[a] N, number of progenies evaluated; h_1^2, heritability on a plot basis; h_2^2, heritability on an entry mean basis; pc, plant cane; r, ratoon; gh, greenhouse

[b] All correlations are significant at $P = 10^{-4}$

Fig. 1A, B Representation of rust susceptibility measures on two-dimensional plots in population P1. **A** Rust susceptibility observed in field trial 2 on plant cane (*pc*) versus ratoon (r). **B** Rust susceptibility observed on ratoon of field trial 2 versus greenhouse test. Measures in the field were graded visually on a 1 to 9 scale; measures in the greenhouse are the square root of the mean uredinia density observed on the leaves. One individual showing an inconsistant response between the two measures in plot **B** is represented by a ◇

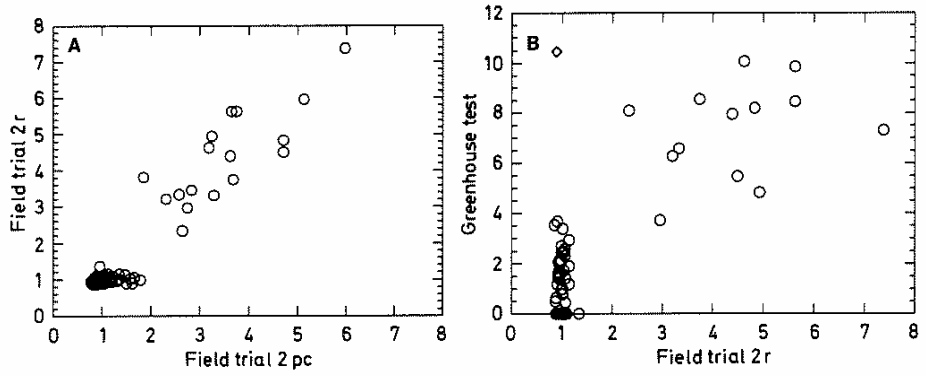


Table 2 QTL detected with Kruskal-Wallis test at the 5% level for the different measurements of rust susceptibility (*pc* plant cane, *r* ratoon, *gh* greenhouse)

Probe[a]	Cosegregation group[a]	D	Kruskal-Wallis test statistic and level of significance			
			Trial 1 pc[c]	Trial 2 pc[c]	Trial 2 r[c]	gh trial[c]
SG12	IV-1	8	7.9*	–	–	–
UMC132	IV-1		7.9*	–	–	–
Adh1	IV-5		10.5*	–	–	–
Adh1	VIII-1	5	–	8.3*	–	–
UMC15	VIII-1	7	–	9.0*	–	–
BNL3.04	VIII-1		–	8.6*	–	–
UMC6	VIII-2	0	9.6*	–	–	10.8**
UMC44	VIII-2	0	9.6*	–	–	10.8**
CDSR125	VIII-2	6	9.6*	–	–	10.8**
SSCIR86	VIII-2		–	–	–	10.8**
UMC137	X-1		8.1*	–	–	–
CDSR29	–		28.4****	27.8****	18.9***	15.5***

* $P \leq 0.0005$, ** $P \leq 0.0001$, *** $P \leq 10^{-4}$, **** $P \leq 10^{-5}$
[a] Grivet et al. (1996)
[b] Distance (in centi Morgans) between linked markers
[c] pc: plant cane; r: ratoon, gh: greenhouse

RFLPs associated with rust resistance loci

In a first round of analysis, the four measures of rust susceptibility were considered as quantitative traits, and quantitative trait loci (QTLs) were searched for using the test of Kruskal-Wallis. With respect to the high number of tests performed, a threshold of $P = 0.005$ was retained to determine significant associations. For all four measures, a highly significant association ($P < 0.0001$) was found with marker *CDSR29-H5* (fifth largest fragment in the *Hin*dIII profile), which was revealed by probe *CDSR29* (Table 2). This marker as well as the other four simplex markers revealed by probe *CDSR29* were not involved in any cosegregation group, thus the QTL could not be positioned on the composite map of 'R570' (Grivet et al. 1996). Five other putative QTLs of smaller effect were found associated with one or two specific measures of rust susceptibility (Table 2). Considering the high number of marker-trait associations investigated, they may include artefacts. However, it is noteworthy that one of those minor QTLs repeatedly arose in two of the four trials. This QTL was located along cosegregation group VIII-2.

Table 3 Contingency table showing linkage between the putative resistance major gene and marker *CDSR29-H5* of probe *CDSR29* in the selfed progeny of 'R570' on population P1, population P2 and on the pooled population

Rust resistance major gene	Marker *CDSR29-H5*			
	Present	Absent	Missing data	Total
Present	96[a] (40[b], 56[c])	4 (3, 1)	2 (2, 0)	102 (45, 57)
Absent	9 (2, 7)	31 (13, 18)	4 (3, 1)	44 (18, 26)
Total	105 (42, 63)	35 (16, 19)	6 (5, 1)	146 (63, 83)

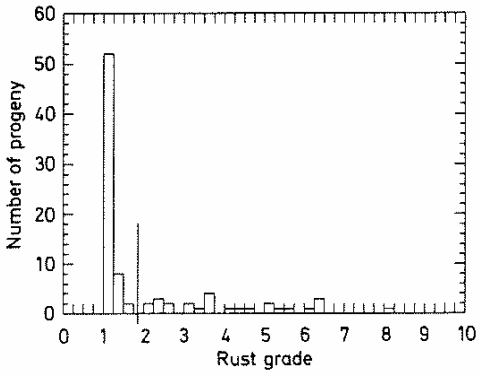
[a] Pooled data on populations P1 and P2
[b] Contribution of population P1
[c] Contribution of population P2

We then investigated linkage of the putative major gene, graded in a presence versus absence fashion, with the 439 markers of the map and found a strong linkage with marker *CDSR29-H5*. The χ^2_L test, which measures linkage independently of segregation distortions with 1 *df* (Mather 1957), was $\chi^2_L = 38.6$ ($P < 0.00001$). The distance between the two Mendelian factors was 8.8 cM with the Haldane mapping function.

Confirmation of the linkage data on another progeny sample

The P2 progeny, consisting of 83 clones, was observed in the field in 1995, and the clones were scored on the 1–9 scale for rust susceptibility. Keeping in mind the possible existence of a major resistance gene, we considered the 63 clones with a score lower than 2 to be highly resistant, while the 26 clones with a grade higher than or equal to 2 were considered to be susceptible (Fig. 2). The ratio of resistant versus susceptible clones was not differ-

Fig. 2 Distribution of rust susceptibility scores in population P2. The *vertical bar* represents the threshold retained to distinguish between individuals bearing the resistance gene and those that do not have it



ent from 3:1 ($\chi^2 = 1.77$). The scoring of marker *CDSR29-H5* on the same individuals permitted us to confirm the linkage with the putative rust major gene ($\chi_2 = 51.0$, distance of 11.1 cM). The χ^2 test measuring discrepancy with 1 *df* between the recombination rate obtained with the two bodies of data (Mather 1957), P1 and P2, was not significant ($\chi = 0.054$). Rust and marker data of P1 and P2 were then pooled, giving a $\chi^2_L = 89.6$ and a genetic distance of 9.5cM between the putative major gene and marker *CDSR29-H5* (Table 3).

Discussion

The present study revealed the presence of a probable simplex major gene conferring resistance to rust disease in sugarcane cv 'R570' and located this gene at 10 cM from an RFLP marker. The existence of a major factor in this variety had already been suggested in a preliminary report of experiments conducted in Mauritius (Saumtally et al. 1994). Our results established its existence with a high level of confidence on the basis of the following:

- characterisation of progeny individuals with RFLP markers identified valid self-progeny clones of 'R570'. This is important, for the lack of an efficient control of pollination is a common source of disturbance in genetic studies on sugarcane;
- the progenies were evaluated in several experiments, and in all of these high heritabilities and high mutual correlations were apparent. Scoring in the field and in the greenhouse varied for only 1 progeny clone, which expressed rust susceptibility only in the greenhouse trial. This discrepancy will require further investigation. It may be due to genotype × environment interaction, for rust susceptibility is largely influenced by environmental factors such as soil (Anderson et al. 1990), climate (Peros et al. 1993) and plant age (Albuquerque 1958). A mislabelling can, however, not be excluded;
- the segregation ratio fitted very well with the expected 3:1 ratio for a dominant simplex gene;
- linkage with a DNA marker was established with a high level of confidence ($\chi^2_L = 89.6$). This permitted us to associate the resistance versus susceptibility of progeny clones with the presence versus absence of a specific chromosomal segment. Even when rust susceptibility was treated as a quantitative trait, the association with the marker was very highly significant for all measures, indicating the presence of a QTL with a very large effect.

Beyond the segregation of the major gene, a large rust susceptibility level variation still existed in the susceptible progeny class. The high heritability on a plot basis suggests that the origin of this variation is at least partially genetically determined. It could be explained by the segregation of QTLs with small effects, such as the one borne by cosegregation group VIII-2. This marginal polygenic variation may explain why a few progeny clones were difficult to classify as resistant or susceptible. A significant genotype × year interaction was noticed in trial 2 (data not shown), indicating that clone ranking for susceptibility can be affected to some extent by the environment (physiological or ecological since year effect is superimposed with ratoon effect in our trial). This could explain why the clear-cut segregation for rust susceptibility appeared more clearly when two measurements were taken simultaneously. When clones do not bear the major gene but have favorable alleles at QTL with small effects, they may appear as resistant in one given trial, whereas the susceptibility might be revealed when several observations are available.

Robinson (1976) considered that vertical pathosystems do not exist in sugarcane, and he stressed that oligogenically inherited resistance has never been recorded in this crop. Since pathogenic races may exist in *P. melanocephala*, the identification of a major gene raises the question of whether it corresponds to a gene-for-gene relationship. The genetics of rust resistance has been very well documented in maize and to a lesser extent in sorghum, two diploid crops belonging to the Andropogoneae tribe, like sugarcane (Hooker 1985). Main specific rust pathogens of maize and sorghum belong to the *Puccinia* genus (respectively, *P. sorghi* and *P. purpurea*). In both species, a general polygenic resistance and a monogenic pathogen race-specific resistance have been characterised. The analogy with our results is noteworthy. Nevertheless, the Flor (1971) gene-for-gene concept has been developed on diploid crop, and a transposition to sugarcane may not be appropriate given the high ploidy level of the cultivars. Indeed, around ten copies of the resistance locus are present in a given clone, each one possibly bearing a race-specific allele. Extending the genetic map of 'R570' will reveal the position of the major gene on a linkage group and thus enable a comparison with the *Rp* maize resistance gene positions on the maize map. If the positions concur, it will be indicative of a probable homology of the gene detected here in sugarcane with the corresponding *Rp* gene of maize.

The development of a polymerase chain reaction (PCR)-based marker closely linked to the major resistance gene could be helpful in screening for rust resistance in large progeny samples although rust resistance is relatively easy to select for on the basis of the phenotype, thanks to its high heritability.

Beyond the specific case of the rust disease, our result is important since it is the first report of a monogenic determinism of disease resistance in the complex polyploid that sugarcane is. Should this exist for the main sugarcane pathogenes for which field trials are usually very laborius, molecular markers could be very useful in assisting breeding.

Acknowledgements We thank Drs. M. Lorieux and P. Letourmy for helpful advice with the statistical analysis, Dr. J. W. Van Ooijen for kindly providing the MAPQTL computer program and Drs. J. da Silva and P. Moore for providing RFLP probes.

References

Albuqueque MJ (1958) Varietal susceptibility to sugarcane rust and its relationship to cane parentage. Deccan Sugar Technol Assoc 15:193–199

Al-Janabi SM, Honeycutt RJ, McClellet M, Sobral BWS (1993) A genetic linkage map of *Saccharum spontaneum* L. "SES 208". Genetics 134:1249–1260

Anderson DL, Raid RN, Irey MS, Henderson LJ (1990) Association of sugarcane rust severity with soil factors in Florida. Plant Dis 74:683–686

Batcha MBGR, Palachamy K (1978) Studies on the inheritance of ligular process in the interspecific cross *S. officinarum* (var. Green Sport) × *S. spontaneum* (CBE). Proc Int Soc Sugar Cane Technol 16:227–231

Comstock JC, Wu KK, Schnell RJ (1992) Heritability of resistance to sugar cane rust. Sugar Cane 6:7–10

D'Hont A, Lu YH, Gonzàlez de Leòn D, Grivet L, Feldmann P, Lanaud C, Glaszmann JC (1994) A molecular approach to unravelling the genetics of sugarcane, a complex polyploid of the andropogoneae tribe. Genome 37:222–230

D'Hont A, Rao PS, Feldmann P, Grivet L, Islam-Faridi N, Taylor P, Glaszmann JC (1995) Identification and characterisation of sugarcane intergeneric hybrids, *Saccharum officinarum* × *Erianthus arundinaceus*, with molecular markers and DNA in situ hybridisation. Theor Appl Genet 91:320–326

D'Hont A, Grivet L, Feldmann P, Rao PS, Berding N, Glaszmann JC (1996) Characterisation of the double genomic structure of modern sugarcane cultivars, *Saccharum* ssp., by molecular cytogenetics. Mol Gen Genet 250:405–413

Da Silva J, Honeycutt RJ, Burnquist W, Al-Janabi SM, Sorrells ME, Tanksley SD, Sobral BWS (1995) *Saccharum spontaneum* L. 'SES 208' genetic linkage map containing RFLP and PCR based markers. Mol Breed 1:165–179

Dean JL, Purdy LH (1984) Races of the Sugar cane fungus *Puccinia melanocephala*, found in Florida. Sugar Cane 1:15–16

Flor HH (1971) Current status of the gene-for-gene concept. Annu Rev Plant Phytopathol 9:275–296

Grivet L, D'Hont A, Roques D, Feldmann P, Lanaud C, Glaszmann JC (1996) RFLP mapping in cultivated sugarcane (*Saccharum* ssp.): Genome organisation in a highly polyploid and aneuploid interspecific hybrid. Genetics 142:987–1000

Hogarth DM, Ryan CC, Skinner JC (1983) Inheritance of resistance to rust in sugarcane-comments. Field Crops Res 7:313–316

Hogarth DM, Ryan CC, Taylor PWJ (1993) Quantitative inheritance of rust resistance in sugarcane. Field Crops Res 34:187–193

Hooker AL (1985) Corn and Sorghum rusts. In: Roelfs AP, Bushnell WR (eds) The cereal rusts. Academic press, London, pp 207–235

Mather K (1975) The measurement of linkage in heredity. Methuen and Co, London

Ooijen Van JW, Sandbrik H, Purimahua C, Vrielink R, Verkerk R, Zabel P, Lindhout P (1992) Mapping quantitative genes involved in a trait assessed on an ordinal scale: a case study with Bacterial Canker in *Lycopersion peruvianum*. In: Yoder JI (ed) Molecular biology of tomato. Davis, Calif. Technomic publishing, Lancaster, USA

Peros JP (1989) Etude quantitative de la resistance de la canne à sucre à *Puccinia melanocephala*. L'Agron Trop 44:123–128

Peros JP (1993) Host environmental influences on infection of sugarcane by *Puccinia melanocephala*. Sugar Cane 2:13–17

Peros JP, Lombard H (1986) Epidemiology of sugar cane rust in Réunion. Sugar Cane 3:15–17

Purdy LH, Liu LJ, Dean JL (1983) Sugarcane rust: a newly important disease. Plant Dis 67:1292–1295

Raghavan TS, Govindaswany S (1956) Sugarcane as a material for genetical studies. Proc Int Soc Sugar Cane Technol 9: 667–694

Robinson RA (1976) Plant pathosystems. Springer, Berlin Heidelbreg New York

SAS Institute (1988) SAS user's guide, version 6, edn. 3, SAS Institute, Cary, N.C.

Saumtally S, Sullivan S, Moutia Y, Autray JC (1994) Assessment of the reaction of progeny to rust (*Puccinia melanocephala* H. and P. SYD) in resistance inheritance studies. In: Bureau of sugarcane experiment station (ed) Fourth ISSCT Pathol Workshop, Brisbane, Australia, p 36

Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples), Biometrika 52:591–611

Sreenivasan TV, Ahloowalia BS, Heinz DJ (1987) Cytogenetics. In: Heinz DJ (ed) Sugarcane improvement through breeding. Elsevier, Amsterdam, pp 211–253

Srinivasan KV, Muthaiyan MC (1965) A note on physiologic races in *Puccinia erianthi* Padw. Khan affecting sugar cane varieties. Proc Int Soc Sugar Cane Technol 12:1126–1128

Tai PYP, Miller JD, Dean JL (1981) Inheritance of resistance to rust in sugarcane. Field Crops Res 4:261–268

Taylor PWJ (1992) Evidence for the existance of a single race of common rust caused by *Puccinia melanocephala*, in Australian sugar cane cultivars. Aust J Agric Res 48:443–450

Taylor PWJ, Croft BJ, Ryan CC (1986) Studies into the effect of sugarcane rust (*Puccinia melanocephala*) on yield. Proc Int Soc Sugar Cane Technol 19:411–419

Protoporphyrinogen Oxidase Inhibitor Herbicide Effects on Pythium Root Rot of Sugarcane, *Pythium* Species, and the Soil Microbial Community

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Accepted for publication 15 September 2004.

ABSTRACT

Daugrois, J. H., Hoy, J. W., and Griffin, J. L. 2005. Protoporphyrinogen oxidase inhibitor herbicide effects on Pythium root rot of sugarcane, *Pythium* species, and the soil microbial community. Phytopathology 95:220-226.

The effects of three protoporphyrinogen oxidase inhibitor herbicides, azafenidin, flumioxazin, and sulfentrazone, on Pythium root rot of sugarcane and the soil microbial community were evaluated in greenhouse experiments. Herbicides were applied as foliar and soil treatments. There were no consistent effects on plant growth or disease parameters. However, some herbicide treatments affected the relative frequency of isolation of *Pythium* spp. from roots and reduced colonization by the pathogenic species *Pythium arrhenomanes*. A comparison of sole carbon source utilization profiles indicated that soil-applied herbicides altered the functional diversity of the soil microbial community, with some variation depending on herbicide used. All three herbicides inhibited the in vitro mycelial growth of *P. arrhenomanes*, *P. aphanidermatum*, and *P. ultimum*. Active ingredients were less inhibitory than formulated product for azafenidin and flumioxazin but not for sulfentrazone.

Sugarcane, interspecific hybrids of *Saccharum*, is a clonally propagated crop from which multiple, successive annual harvests of stalks typically are obtained. Yield can be adversely affected by diverse biological factors, including a soilborne disease complex and weed competition. Pythium root rot, caused primarily by *Pythium arrhenomanes*, is an important component of the root disease complex in more temperate climatic regions (12,17,25). Aboveground obvious disease symptoms are not observed in modern interspecific hybrid cultivars, but reduced tillering and stalk weight can result from rotting of young roots by *Pythium* spp. Sugarcane often is grown as a monoculture, and evidence suggests that long-term cultivation of sugarcane can result in changes in the soil microbial community that are detrimental to plant growth (8,20,21).

Weeds are controlled through tillage and herbicide applications. The effects of six herbicides routinely applied to sugarcane in Louisiana on plant growth, root rot, and *Pythium* mycelial growth were evaluated in a previous study (4). Two herbicides, pendimethalin and atrazine, inhibited the mycelial growth of *P. arrhenomanes*, but neither reduced root rot severity in greenhouse experiments. Three herbicides, glyphosate, pendimethalin, and terbacil, were injurious to sugarcane and increased root rot severity. Metribuzin had no effect on root rot or mycelial growth.

Since the previous herbicide study, three protoporphyrinogen oxidase (PPOase) inhibitor herbicides, azafenidin (30), flumioxazin, and sulfentrazone (30), have been evaluated for postemergence weed control in Louisiana. Herbicides that inhibit PPOase result in the generation of singlet oxygen radicals that destroy lipids in cell membranes. A variety of PPOase inhibitor herbicides have been demonstrated to reduce the severity of Sclerotinia stem rot of soybean (3,22,23), reduce soybean cyst nematode reproduction (18), and reduce severity of Rhizoctonia aerial blight of soybean (1). Reduced disease severity is associated with enhanced defense mechanisms, including elicitation and accumulation of phytoalexin and mimicry of the hypersensitive response (14,22,23).

There has been long-standing interest in the effects of herbicides on soil microflora (10,26). Sole carbon source utilization profiles (7) have been used to evaluate soil microbial community functional diversity in agricultural cropping systems (5,6,16,19). PPOase inhibitor herbicides are subject to soil adsorption, but their possible effects on soil microflora are unknown.

Field observations suggested the possibility of enhanced sugarcane growth resulting from azafenidin treatments without weed competition or visible disease symptom differences between treated and nontreated portions of fields. These observations prompted a study to evaluate the effects of PPOase inhibitor herbicides on *Pythium* spp. and root rot in sugarcane. Due to the complex nature of soilborne disease in sugarcane, possible effects of the PPOase inhibitor herbicides on the soil microbial community also were examined.

MATERIALS AND METHODS

Two experiments were conducted in the greenhouse during fall 2000 and winter 2001. Maximum and minimum average temperatures were 28 and 17°C for the fall experiment and 30 and 17°C for the winter experiment. The soil used in both experiments was a Commerce silt loam collected from a sugarcane field at the St. Gabriel Research Station of the Louisiana State University Agricultural Center in Iberville Parish. Soil was sieved through a 1-cm-mesh screen and mixed. A portion of soil was steam sterilized by exposure to steam for 24 h in metal trays (50 by 70 by 8 cm). Nonsterile or steam-treated soil then was mixed with steam-treated sand to a final field soil/sand ratio of 1:1 (vol/vol). Untreated field soil mixed with steam-treated sand will be

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DOI: 10.1094/PHYTO-95-0220

referred to as field soil (FS), and the steamed field soil–sand mixture will be referred to as steamed field soil (SFS). A portion of SFS was infested with *P. arrhenomanes* inoculum and will be referred to as *P. arrhenomanes*-infested SFS (SFS+P). The *P. arrhenomanes* inoculum was prepared and added to soil as described previously (12). Inoculum consisted of mycelium and reproductive structures produced in a mixture of vermiculite, oat, and V8 vegetable juice that was added to the potting medium after rinsing at a ratio of 1:50.

Herbicide effects were evaluated on sugarcane transplants grown in clay pots. Four-week-old plants of cv. LCP 85-384 with three to four leaves, grown from single-bud cuttings, were transplanted in 15-cm-diameter clay pots (one plant per pot). Two herbicide applications were made: foliar application and soil application. For the herbicide leaf application treatment, herbicides were applied to plant foliage just before transplantation. Roots and soil were protected from contact with herbicide spray by covering soil with plastic film. For the herbicide soil application treatment, herbicides were applied to the soil surface just after plants were transplanted in clay pots. Foliage was protected from herbicide spray by wrapping the leaves in plastic film. Commercial formulations of three herbicides, azafenidin (Milestone DF 80; DuPont De Nemours & Co. Inc., Newark, DE), flumioxazin (Valor DF 50; Valent USA Corporation, Walnut Creek, CA), and sulfentrazone (Spartan DF 75; FMC Corporation, Philadelphia) were applied at the recommended rates (0.57, 0.28, and 0.285 kg a.i./ha for Milestone, Valor, and Spartan, respectively) and 1/10 of the recommended rates in both experiments. Azafenidin and sulfentrazone are in the aryl triazinone chemical family, and flumioxazin is an N-phenylphthalimide. After extensive testing, azafenidin was not labeled for commercial use. Control treatments with no herbicide application were included for comparison with herbicide treatments for all soil types. The herbicides were applied to plants in a spray chamber. The sprayer was calibrated to deliver 140 liter ha^{-1} at a pressure of 1.65 bar.

Five plants per treatment were randomly placed on two greenhouse benches. On each bench, the three soil types, FS, SFS, and SFS+P, were separated by clear plastic film barriers to prevent cross contamination. Plants were watered daily to maintain the soil moisture level near field capacity. Plants were fertilized every 2 weeks with a water-soluble fertilizer mixture (N-P-K, 20-20-20). Plant injury caused by herbicides was recorded at 15, 30, and 45 days after herbicide treatments, using a 0-to-5 rating scale, in which 0 = no damage, 1 = <5% of the leaf surface exhibiting chlorosis and necrosis, 2 = >5 to 20% injury, 3 = >20 to 50% injury, 4 = >50 to 90% injury, and 5 = plant death. Plant injury data were analyzed after calculation of the area under the herbicide damage progress curve (AUHDPC).

Evaluation of herbicide effects on root rot, plant growth, and *Pythium* spp. colonization. The effects of herbicides were evaluated 8 to 10 weeks after treatment. Plant data recorded included total shoot number per plant, root system fresh weight, and total shoot dry weight. In addition, a visual rating of root system reduction and root rot in comparison with the appropriate control was made for each plant on a scale of 1 to 4, in which 1 = normal appearance, no discoloration or reduction of lateral root system; 1.5 = very mild disease, less than 10% root discoloration or reduction in lateral root system; 2 = mild disease, 10 to 25% damage, 2.5 = moderate disease, 26 to 50% damage; 3 = severe disease, 51 to 75% damage; 3.5 = very severe disease, 76 to 90% damage; and 4 = complete disease.

The extent of root colonization by *Pythium* spp. also was determined. Six root samples, 5 cm in length, were collected from each of five plant replicates per treatment. Root segments were washed in deionized water on a rotary shaker, blotted dry, placed in a 9-cm-diameter petri dish, and immersed in molten selective medium at 50°C. The medium contained 10 g each of corn meal agar and agar autoclaved in 1 liter of distilled water. It was amended after cooling to 50°C with 15 mg of pentachloronitrobenzene dissolved in 95% EtOH, 250 mg of ampicillin and 10 mg of rifampicin dissolved in 1 ml of distilled water, and 0.4 ml of pimaricin (10 mg a.i./liter). The agar was allowed to harden, and the number of cm of root colonized by *Pythium* spp. was determined after 24 and 48 h at room temperature. Forty isolates were collected from infected roots for identification from each FS treatment.

Pythium isolates from root samples were identified as described previously (4). Isolates were transferred to individual petri dishes containing V8 medium (200 ml of V8 vegetable juice, 2 g of $CaCO_3$, and 17 g of Bacto agar). After 24 to 48 h of growth, six 3-mm-diameter agar plugs containing actively growing mycelium were transferred into a 5.5-cm-diameter petri dish and flooded with 5 to 7 ml of filter-sterilized soil extract. After 2 to 3 days at room temperature, identification of *Pythium* spp. was accomplished by microscopic examination of reproductive structures using the key of Van der Plaats-Niterink (29).

Characterization of soil microbial communities. To determine herbicide effects on the soil microbial community, soil samples from each full rate soil herbicide treatment were sampled as described by Fantroussi et al. (5) with some modifications. Briefly, soil samples were collected with a soil core at a depth of 3 to 5 cm from four different pots, pooled, and mixed. Three subsamples of 5 g of soil were taken from the pooled soil before extraction of microorganisms from soil. Isolation of microorganisms was performed by modifying the methods of Smalla (27) and Staddon et al. (28). Subsamples were suspended separately in 25 ml of sterile saline solution (0.85% NaCl), and agitated for 1 h on a rotary shaker at 200 rpm at room temperature. The soil suspension then was centrifuged for 10 min at 500 × *g* to remove soil and root particles. The supernatant was transferred to a sterile centrifuge tube. Microorganisms were washed twice with 20 ml of sterile saline solution (10,000 × *g* for 10 min) and then suspended in 40 ml of sterile saline solution. GN2 and GP2 Biolog (Biolog Inc., Hayward, CA) plates for gram-negative and -positive bacteria were inoculated with 140 µl of washed microorganisms per well and incubated at 28°C for 5 days. Absorbance readings were made each day with a microplate autoreader at 600 nm with blanking done on well A1. The experiment was conducted twice, 4 and 6 weeks after herbicide treatments.

For both experiments, results from the third day for a total of 128 substrates (maximum values for duplicated substrates in GN2 and GP2 plates were used) were compared for the two experiments combined. The number of variables was reduced to 69 by discarding single carbon sources with values below 0.5 and, as recommended by Glimm (9), variables with well means above 2.0 also were discarded. Standardization of optical density (OD) values was performed as proposed by Garland and Mills (7). Briefly, each OD value from one plate was divided by the average well color development (AWCD) of the plate. AWCD was used as a general indicator of microbial activity.

Evaluation of herbicide effects on in vitro mycelial growth of three *Pythium* spp. Cornmeal agar (Difco Laboratories, Detroit) was amended after sterilization with the commercial formulation and the pure active ingredient of azafenidin, flumioxazin, and sulfentrazone at 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0 times the recommended application rates (0.57, 0.28, and 0.28 kg a.i. ha^{-1}, respectively). The concentrations of herbicide active ingredient for rate one were 3, 1.5, and 1.5 g $liter^{-1}$ for azafenidin, flumioxazin and sulfentrazone, respectively.

Agar plugs, 3 mm in diameter, were removed from the margins of actively growing colonies of *P. arrhenomanes*, *P. aphanidermatum*, and *P. ultimum*, placed in the center of herbicide-amended plates, and incubated at 25°C. Radial growth of mycelium from the center was recorded at 24 and 48 h. A mycelial growth rate per 24 h then was calculated. Each treatment was replicated four times. The experiment was conducted twice.

Statistical analyses. SAS (version 8.2; SAS Institute Inc., Cary, NC) was used for data analysis. Herbicide effects on Pythium root rot and plant growth were analyzed as follows. All results from one type of soil and one herbicide rate were combined and analyzed together with herbicide and application as fixed effects and experiment as a random effect. SAS mixed procedure was used to analyze the herbicide application treatment effects, and least square means were used to compare treated plants to nontreated control plants. Percent of root length colonized by *Pythium* spp. was analyzed after angular transformation (x = arcsine [sqrt (L/30)]) where L = length of root colonized by *Pythium* spp. out of the 30 cm plated. Normality of the data was controlled under univariate procedure. Root rot severity and AUHDPC data were analyzed by GLM procedure with herbicide and application as combined sources of variation and experiments as repeated measures. Single carbon source utilization profiles were analyzed by principal component analysis using the covariance matrix.

RESULTS

Herbicide effects on plant growth and Pythium root rot. Recommended rate herbicide treatments were applied separately on leaves of cv. LCP 85-384 and on soil. Azafenidin applied to either the leaves (HLA) or the soil (HSA) was phytotoxic to plants (Fig. 1A). Plant death resulted from soil application in SFS, and extensive leaf necrosis and reduced plant growth was observed for the other soil types. Other herbicide treatments produced only minor necrosis on SFS+P and FS plants, and damage decreased during the experiment (Fig. 1A). Due to plant deaths, results for azafenidin applied to soil at the full rate were discarded from the statistical analysis.

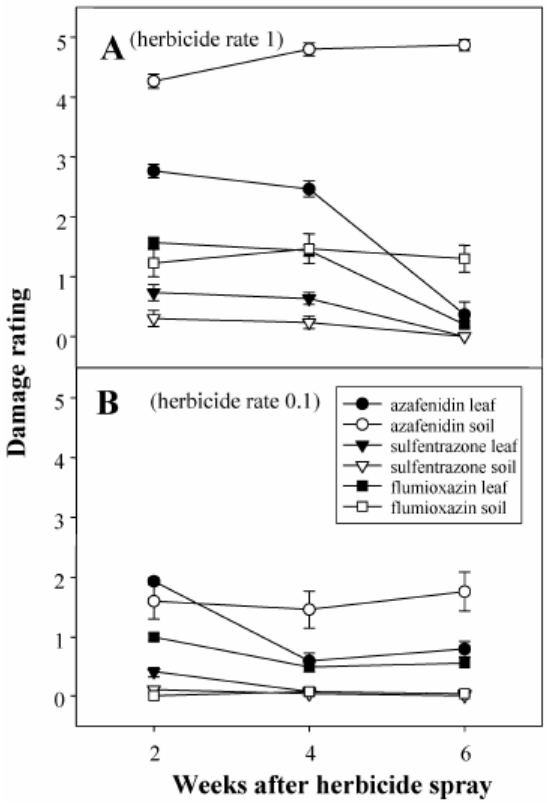

Fig. 1. Evaluation of phytotoxicity symptoms caused by herbicide treatments at two rates, **A**, full recommended rate (1.0) and **B**, 1/10 the full rate (0.1). Results of two experiments were combined. Data points represent means of each herbicide treatment for each date of rating, and error bars are the standard error of the mean. Azafenidin was not applied to soil at the full rate in experiment two.

Overall, no effect of the full rate of herbicide application either on soil or leaf was observed on shoot weight for either FS or SFS+P plants, and only one treatment, flumioxazin applied to soil, was significantly different from the control (Table 1). Compared with control plants, two full-rate herbicide treatments had a negative effect on root growth. Flumioxazin applied to soil reduced root weight by 29 and 36% on FS and SFS+P plants, respectively, and also caused a 44% reduction for SFS plants (Table 1). Azafenidin applied to leaves at the full rate also reduced root weight of FS and SFS plants by 30 and 24%, respectively. All three herbicides had a negative effect on root weight of SFS plants when applied at full rate to soil (Table 1). For full-rate herbicide treatments, no significant effect on root colonization by total *Pythium* spp. was observed; however, colonization by *P. arrhenomanes* was reduced by sulfentrazone and flumioxazin applied to soil in FS (Table 1). There was no effect on root rot severity rating (data not shown).

When herbicides were applied at the 0.1 rate, the only negative herbicide effects on plant growth were due to azafenidin applied to soil in FS and SFS and flumioxazin applied to soil in SFS+P (Table 2). At rate 0.1 of the recommended herbicide rate, visible damage observed on plants was reduced (Fig. 1B). All other root and shoot weights for herbicide-treated plants and control plants were similar, except sulfentrazone applied to soil increased root weight (P = 0.056) in SFS+P (Table 2). There were variable effects of herbicide treatments on *Pythium* spp. root colonization (Table 2). Colonization by *Pythium* spp. and *P. arrhenomanes* was decreased by foliar application of flumioxazin in FS (P = 0.095 and 0.078, respectively) and colonization by *P. arrhenomanes* was decreased by sulfentrazone applied to leaves in SFS+P (P = 0.003). However, colonization by *Pythium* spp. was increased by sulfentrazone soil application in FS. For flumioxazin and sulfentrazone applied to SFS+P and FS plants, root colonization by *Pythium* spp. was significantly higher for rate 0.1 applied to soil than for rate 0.1 applied to leaves. Only flumioxazin applied to leaves in SFS+P significantly reduced the root rot severity rating (Fig. 2).

Herbicide effects on *Pythium* spp. isolation from sugarcane roots. *Pythium* spp. were identified and quantified after isolation in each experiment. Results showed that azafenidin applied to leaves at the full rate (P = 0.036) and sulfentrazone and flumioxazin applied to soil at the full rate (P = 0.017) on FS plants significantly reduced the percentage of isolates that were *P. arrhenomanes* (Fig. 3A). The percentage of isolates consisting of other species increased from 29% (control plants) to 67% for soil-applied flumioxazin and 60% for soil-applied sulfentrazone and azafenidin applied to leaves at the full rate. Other *Pythium* spp. identified on FS were *P. spinosum*, *P. graminicola*, *P. irregulare*, and *P. torulosum* (Fig. 3). Some herbicides appeared to change profiles of species isolated from infected roots. Sulfentrazone and flumioxazin applied to soil at the full rate and sulfentrazone applied to soil at rate 0.1 significantly enhanced occurrence of *P. tolurosum* (P = 0.018, 0.033, and 0.023, respectively, for the three herbicide applications), and the percentage of isolates identified as *P. spinosum* was significantly higher for sulfentrazone and azafenidin applied to soil at rate 0.1 (P = 0.013 and 0.010) (Fig. 3B).

Herbicide effects on the soil microbial community. Soil microbial community functional diversity evaluated by sole carbon substrate utilization profile was affected by herbicide treatment in FS. No differences between treatments were observed for AWCD values calculated for each Biolog plate. Means for AWCD values ranged from 0.71 for azafenidin treatment to 0.87 for the untreated control. The first principal component (PC1), representing

27% of the standardized variance of carbon source utilization, corresponded mainly to differences between sampling dates. The difference between sampling dates also was observed for PC2. On the other hand, PC2 clearly separated the microbial community under flumioxazin treatment from communities affected by the two other herbicide treatments (Fig. 4A). PC3 and PC4 were not representative of variation between sampling dates. However, PC3 differentiated microbial communities for the azafenidin and control treatments from microbial communities for the sulfentrazone and flumioxazin treatments. PC4 differentiated microbial communities under all three herbicide treatments from the microbial community of control soil (Fig. 4B). Carbon substrates most responsible for explaining variability in substrate utilization were carbohydrates (Table 3).

Herbicide effects on mycelial growth of *Pythium* spp. in vitro. Commercial formulations of all herbicides reduced *P. arrhenomanes*, *P. aphanidermatum*, and *P. ultimum* mycelial growth even at the lowest herbicide concentration (Fig. 5). The lowest concentration used was 0.0187, 0.015, and 0.01 g liter^{-1} for azafenidin, flumioxazin, and sulfentrazone, respectively. Those

TABLE 1. Effects of recommended-rate herbicide treatments on root colonization by *Pythium* spp. and root and shoot weight[a]

Soil[b]	Herbicide	Application	Length of root colonized (out of 30 cm)		Root weight (g)	Shoot weight (g)[d]
			Pythium spp.	*P. arrhenomanes*[c]		
FS	Control	None	6.3	3.9	10.4	4.1
FS	Azafenidin	Leaf	8.5	2.6*	7.3**	3.4
FS	Sulfentrazone	Leaf	4.3	2.7*	10.3	4.2
FS	Flumioxazin	Leaf	10.2	5.9	8.3*	3.9
FS	Sulfentrazone	Soil	4.7	2.2**	9.5	3.6
FS	Flumioxazin	Soil	4.5	1.2***	7.4**	3.7
FS	Azafenidin	Soil[e]	…	…	1.2	0.7
SFS+P	Control	None	…	20.6	10.4	4.5
SFS+P	Azafenidin	Leaf	…	19.8	9.1	3.9
SFS+P	Sulfentrazone	Leaf	…	16.9	10.3	4.0
SFS+P	Flumioxazin	Leaf	…	24.0	10.0	4.3
SFS+P	Sulfentrazone	Soil	…	19.1	9.6	3.8
SFS+P	Flumioxazin	Soil	…	19.7	6.6**	3.1**
SFS+P	Azafenidin	Soil[e]	…	…	0.6	0.3
SFS	Control	None	…	…	33.9	7.6
SFS	Azafenidin	Leaf	…	…	25.6*	7.6
SFS	Sulfentrazone	Leaf	…	…	29.2	8.2
SFS	Flumioxazin	Leaf	…	…	28.4	7.6
SFS	Sulfentrazone	Soil	…	…	25.5**	7.2
SFS	Flumioxazin	Soil	…	…	19.0***	6.4**
SFS	Azafenidin	Soil[e]	…	…	0	0

[a] Values represent means from two experiments combined. Probability for treatment lsmean = control plant lsmean (P [>|t|]< value) after arcsine transformation of data: *, **, and *** = $P < 0.1$, 0.05, and 0.01, respectively.
[b] FS = field soil, SFS+P = steamed soil with *Pythium arrhenomanes* inoculum, and SFS = steamed field soil.
[c] Root colonization by *P. arrhenomanes* was estimated after *Pythium* sp. identification.
[d] Statistical analyses were made after square root transformation of data.
[e] Not included in statistical analysis. Means indicated are from trial one; treatment was not repeated due to severe plant damage.

TABLE 2. Effects of rate 0.1 herbicide treatments on root colonization by *Pythium* spp. and root and shoot weight[a]

Soil[b]	Herbicide	Application	Length of root colonized (out of 30 cm)		Root weight (g)	Shoot weight (g)[d]
			Pythium spp.	*P. arrhenomanes*[c]		
FS	Control	None	7.1	4.8	10.1	4.1
FS	Azafenidin	Leaf	9.0	5.2	9.5	4.0
FS	Sulfentrazone	Leaf	5.5	3.7	10.2	4.2
FS	Flumioxazin	Leaf	3.3*	2.0*	10.8	4.2
FS	Azafenidin	Soil	8.9	5.6	8.2**	3.7
FS	Sulfentrazone	Soil	17.1***	8.9	10.1	4.1
FS	Flumioxazin	Soil	8.8	4.0	9.57	4.0
SFS+P	Control	None	…	20.6	10.4	4.3
SFS+P	Azafenidin	Leaf	…	18.7	11.4	4.4
SFS+P	Sulfentrazone	Leaf	…	9.2**	10.7	4.7
SFS+P	Flumioxazin	Leaf	…	18.1	11.3	4.3
SFS+P	Azafenidin	Soil	…	14.7	11.3	4.3
SFS+P	Sulfentrazone	Soil	…	18.3	12.7*	4.8
SFS+P	Flumioxazin	Soil	…	24.0	8.6	3.3*
SFS	Control	None	…	…	33.9	7.6
SFS	Azafenidin	Leaf	…	…	28.6	8.2
SFS	Sulfentrazone	Leaf	…	…	31.9	9.6***
SFS	Flumioxazin	Leaf	…	…	29.4	8.9*
SFS	Azafenidin	Soil	…	…	11.6***	4.2*
SFS	Sulfentrazone	Soil	…	…	30.0	8.2
SFS	Flumioxazin	Soil	…	…	33.3	7.6

[a] Values represent means from two experiments combined. Probability for treatment lsmean = control plant lsmean (P [>|t|]< value) after arcsine transformation of data: *, **, and *** = $P < 0.1$, 0.05, and 0.01, respectively.
[b] FS = field soil, SFS+P = steamed soil with *Pythium arrhenomanes* inoculum, and SFS = steamed field soil.
[c] Root colonization by *P. arrhenomanes* was estimated after *Pythium* sp. identification.
[d] Statistical analyses were made after square root transformation of data.

concentrations correspond to a 200-fold dilution of the recommended herbicide rates for field treatment. At this concentration, *P. arrhenomanes* growth was reduced by 67, 34, and 36% by azafenidin, flumioxazin, and sulfentrazone, respectively. Total growth inhibition of *P. arrhenomanes* was observed at the recommended rate (rate 1) for all three herbicides. Corresponding concentrations of active ingredient alone also were tested. At the lowest concentration used, 0.015, 0.0075, and 0.0075 g a.i. liter^{-1} for azafenidin, flumioxazin, and sulfentrazone, respectively, *P. arrhenomanes* growth was reduced by 54, 12, and 39% (Fig. 5A).

For *P. aphanidermatum*, the growth reduction observed at the 200-fold dilution of the recommended herbicide rate (rate 1) ranged from 12 to 54% depending on the herbicide. Complete inhibition of *P. aphanidermatum* was observed only for azafenidin at a 2× concentration of the recommended rate. Maximum growth inhibition for the two other herbicides ranged from 85 to 90% at the 2× concentration of the recommended rate (Fig. 5B). For *P. ultimum*, growth reductions observed at the full rate 200-fold dilution of the herbicides ranged from 5 to 43% depending on herbicide. Complete inhibition of *P. ultimum* was observed for azafenidin for the recommended rate concentration. Maximum growth inhibition for the two other herbicides occurred at a 2× concentration of the recommended rate. The reductions were 62 and 99% for flumioxazin and sulfentrazone, respectively (Fig. 5C). Mycelial growth inhibition caused by the corresponding active ingredient concentrations generally was less for azafenidin and flumioxazin but not sulfentrazone (Fig. 5).

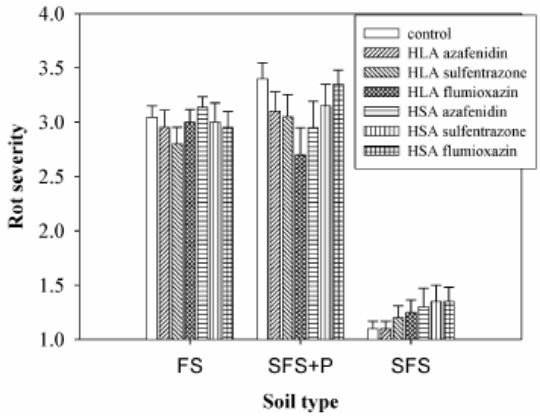

Fig. 2. Effect of herbicides applied at rate 0.1 on root rot severity rating estimated on a 1-to-4 scale. Bars represent means of results from two experiments combined, and error bars are the standard error of the mean. FS = field soil, SFS = steamed field soil, and SFS+P = steamed soil with *Pythium arrhenomanes* inoculum. HSA = herbicides applied to soil, and HLA = herbicide applied to leaves.

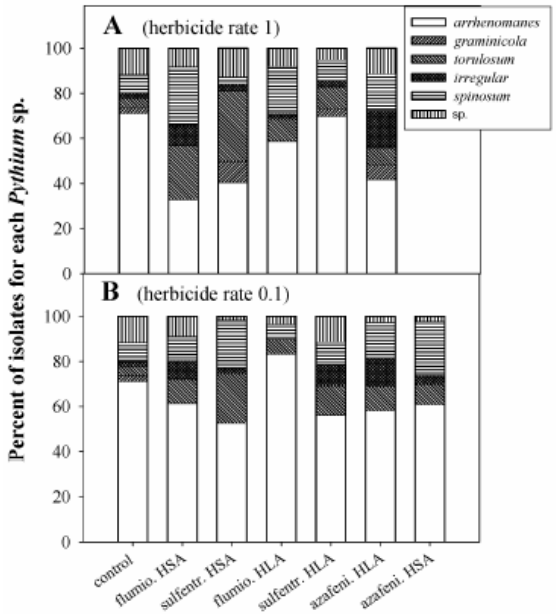

Fig. 3. Frequency of isolation for five *Pythium* spp. and unidentified *Pythium* spp. from roots of sugarcane grown in field soil after herbicides were applied at **A**, the recommended rate and **B**, 1/10 of the recommended rate. Control = nontreated plants, flumio = flumioxazin, sulfentr = sulfentrazone, azafeni = azafenidin, HSA = herbicides applied to soil, and HLA = herbicide applied to leaves.

DISCUSSION

The first research investigating the effects of PPOase inhibitor herbicides on Sclerotinia stem rot of soybean was stimulated by

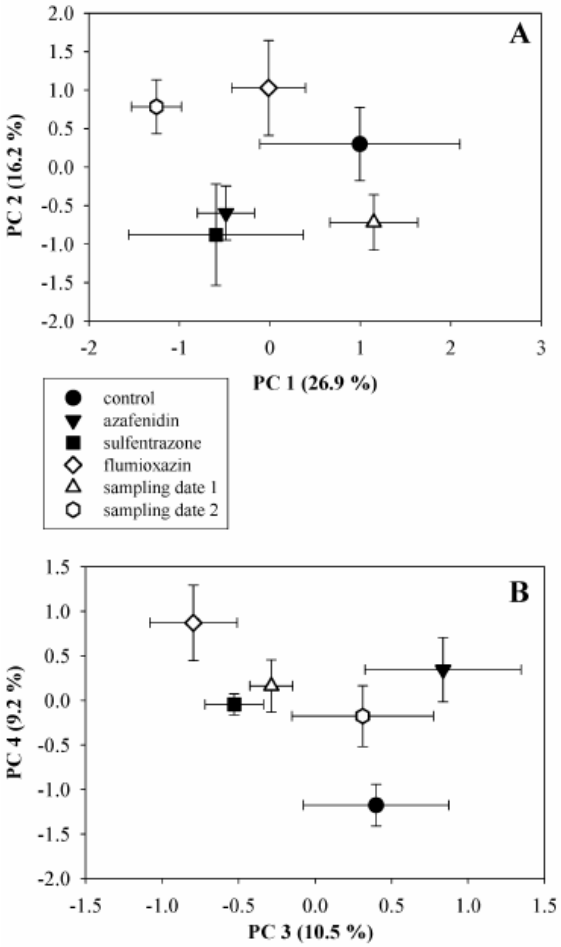

Fig. 4. Effects of three herbicides on soil microbial community functional diversity analyzed by principal component (PC) analysis performed on sole carbon source utilization profiles from treatments with herbicides applied to soil at the recommended rate. **A**, First and second PC axis; **B**, third and fourth PC axis. The two dates of sampling were combined for the herbicide treatment ordination plot of PC and herbicide treatments were combined for each date of sampling ordination plot of PC. Error bars represent standard error of the mean. Values in parentheses indicate the percent of total variation accounted for by each principal component axis.

field observations of reduced symptom severity and enhanced growth for treated plants (3). *Sclerotinia* spp. reside in the soil, but the disease affects aboveground portions of the plant. Similar field observations of increased growth occasionally were made for sugarcane plants treated with azafenidin, but changes in foliar disease symptoms were not observed. This suggested the possibility that plant growth increases might be due to the suppression of root disease. Pythium root rot adversely affects plant growth of sugarcane in Louisiana (12,25); therefore, research was conducted to evaluate possible effects of PPOase inhibitor herbicides on *Pythium* spp. and root rot.

Research evaluating the effects of this class of herbicides on soilborne pathogens and root diseases has been conducted. PPOase inhibitor herbicides applied to soybean plants, but not to soil, resulted in the production and accumulation of a phytoalexin, glyceollin, in roots, and reduced reproduction of the soybean cyst nematode was observed (18). However, herbicide application did not increase yield (2). Lactofen, a PPOase inhibitor herbicide in the diphenyl ether chemical family, did not reduce severity of Rhizoctonia root rot and damping-off in soybean (11). In contrast, acifluorfen, another diphenyl ether herbicide, reduced Rhizoctonia foliar blight intensity in soybean (1).

In this study, there were no consistent beneficial effects on sugarcane from applications of three PPOase inhibitor herbicides, azafenidin, flumioxazin, and sulfentrazone. Herbicide treatments resulted in plant injury. This is crucial for the induction of resistance, but some treatments resulted in significant reductions in growth of plants in SFS. It may be that the duration of the experiments was not long enough for plants to recover from herbicide phytotoxicity effects. The artificial growing conditions inherent in greenhouse experiments also may have prevented the detection of treatment effects. The 0.1 rate was included to investigate the effects of less injurious treatments; however, no significant increases in plant growth resulted from these treatments.

There also were no consistent effects from herbicide treatments on disease parameters. However, some of the results provided evidence suggesting herbicide treatment effects on root rot severity. Flumioxazin applied to leaves at the 0.1 rate in SFS with *P. arrhenomanes* reduced the root rot severity rating, and sulfentrazone applied to leaves at the 0.1 rate in SFS+P reduced root colonization by *P. arrhenomanes*. In addition, multiple treatments changed the relative frequency of isolation of different *Pythium* spp. from the roots and reduced the portion of the root system colonized by *P. arrhenomanes*. Previous research (17) suggested that the percentage of *Pythium* spp. colonizing the roots composed by *P. arrhenomanes* is an important determinant of root rot severity in sugarcane.

Herbicides may modify the competitiveness of soil microorganisms, and *Pythium* spp. apparently are very sensitive to PPOase inhibitor herbicides. At a low concentration, all three herbicides tested inhibited mycelial growth of three different plant-pathogenic *Pythium* spp., and *P. arrhenomanes* exhibited the greatest inhibition. *P. ultimum* was less sensitive to flumioxazin than to the other two herbicides. Formulated products of azafenidin and flumioxazin were more inhibitory to mycelial growth of *Pythium* spp. than the active ingredient for each, whereas sulfentrazone formulated product and active ingredient were equally effective. Treatments in which herbicides were applied to the soil surface were most effective in reducing root colonization by *Pythium* spp. However, plants growing in the field produce an extensive root system, and exposure of *Pythium* spp. to direct herbicide toxicity throughout the soil profile where roots develop would be difficult to achieve.

Sole carbon source utilization profiles have been used to characterize microbial communities in soils planted with agricultural

TABLE 3. Most important carbon substrates for explaining variability in substrate utilization among soils treated with different herbicides based on substrate eigenvector value for principal component one (PC1) to PC4[a]

Substrates	PC1	PC2	PC3	PC4
Carbohydrates				
N-Acetyl-D-galactosamine	0.20	0.46	0.11	0.05
N-Acetyl-D-glucosamine	0.18	0.02	0.36	0.02
D-Arabitol	0.14	0.11	0.22	0.16
Cellobiose	0.02	0.23	0.01	0.19
Gentiobiose	0.02	0.28	0.06	0.28
D-Mannose	0.39	0.06	0.01	0.08
D-Melibiose	0.24	0.26	0.42	0.03
β-Methyl-D-glucoside	0.03	0.04	0.09	0.42
D-Psicose	0.14	0.34	0.14	0.02
D-Raffinose	0.16	0.24	0.39	0.29
L-Rhamnose	0.24	0.12	0.06	0.13
Maltotriose	0.18	0.09	0.00	0.21
D-Sorbitol	0.38	0.02	0.18	0.04
Carboxylic acids				
D-Galacturonic acid	0.09	0.08	0.21	0.00
Amide				
Succinamic acid	0.22	0.16	0.17	0.47
Aromatics				
Urocanic acid	0.22	0.02	0.17	0.02

[a] Only eigenvectors with $|x| > 0.20$ for at least one PC are presented.

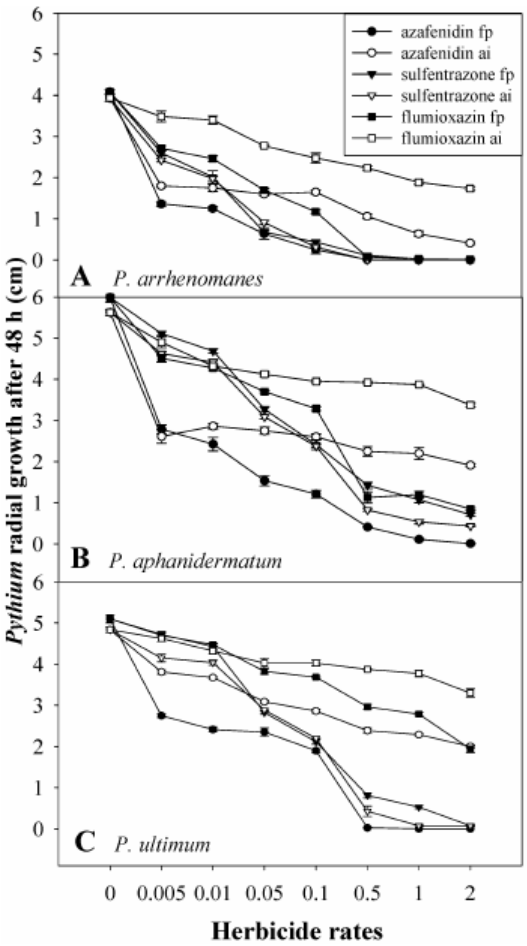

Fig. 5. Herbicide effects on in vitro mycelial growth of three *Pythium* spp.: **A**, *Pythium arrhenomanes*, **B**, *P. aphanidermatum*, and **C**, *P. ultimum*. Rate 1 was the recommended rate and corresponded to an amendment of 3, 1.5, and 1.5 g liter^{-1} of herbicide active ingredient in culture medium for azafenidin, flumioxazin, and sulfentrazone, respectively. Error bars represent the standard error of the mean from two experiments; fp = commercial formulated product of the herbicide and ai = herbicide active ingredient only.

crops and exposed to different treatments (5,16,19). This technique detected alterations in the soil microbial community resulting from treatment with three PPOase inhibitor herbicides, as was observed for phenylurea herbicides (5). The communities in soils treated with the different herbicides also were distinguishable. Major substrates affected were carbohydrates. Carbohydrates were shown to be involved in microbial community differentiation under different potato cropping systems (16), cropping systems that may affect soilborne diseases (15). Research has focused recently on the role of the total microbial community on root health and sugarcane growth and yield (20,24). In addition to possible direct effects of herbicides on *Pythium* spp., alterations in the soil microbial community resulting from exposure to PPOase inhibitor herbicides might affect root disease severity in the field.

Suppression of disease associated with PPOase inhibitor herbicide treatments has been linked to induced resistance mechanisms, such as phytoalexin accumulation or the hypersensitive response (14). Gyceollin accumulation is associated with the expression of resistance to *Phytophthora* spp., organisms closely related to *Pythium* spp., but the effect of herbicides on this association has been studied only with glyphosate (13) and not with PPOase inhibitor herbicides. *Pythium* spp. are nonspecialized pathogens capable of infecting immature root and seedling tissues in multiple hosts. The experimental results with *Rhizoctonia* spp. (11) and *Pythium* spp. suggest that disease resistance that may be induced by herbicides is not effective in root diseases with a low level of specificity between host and pathogen.

LITERATURE CITED

1. Black, B. D., Russin, J. S., Griffin, J. L., and Snow, J. P. 1996. Herbicide effects on *Rhizoctonia solani* in vitro and Rhizoctonia foliar blight of soybean (*Glycine max*). Weed Sci. 44:711-716.
2. Browde, J. A., Pedigo, L. P., Owen, M. D. K., and Tylka, G. L. 1993. Soybean yield and pest management as influenced by nematodes, herbicides, and defoliating insects. Agron. J. 68:601-608.
3. Dann, E. K., Diers, B. W., and Hammerschmidt, R. 1999. Suppression of Sclerotinia stem rot of soybean by Lactofen herbicide treatment. Phytopathology 89:598-602.
4. Dissanayake, N., Hoy, J. W., and Griffin, J. L. 1998. Herbicide effects on sugarcane growth, Pythium root rot, and *Pythium arrhenomanes*. Phytopathology 88:530-535.
5. EL Fantroussi, S., Vershuere, L., Verstraete, W. L., and Top, E. M. 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. Appl. Environ. Microbiol. 65:982-988.
6. Gagliardi, J. V., Buyer, J. S., Angle, J. S., and Russek-Cohen, E. 2001. Structural and functional analysis of whole-soil microbial communities for risk and efficacy testing following microbial inoculation of wheat roots in diverse soils. Soil Biol. Biochem. 33:25-40.
7. Garland, J. L., and Mills, A. L. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon source utilization. Appl. Environ. Microbiol. 57:2351-2359.
8. Garside, A. L., Bramley, R. G. V., Bristow, K. L., Holt, J. A., Magarey, R. C., Nable, R. O., Pankhurst, C. E., and Skjemstad, J. O. 1997. Comparisons between paired old and new land sites for sugarcane growth and yield and soil chemical, physical, and biological properties. Proc. Aust. Soc. Sugar Cane Technol. 19:60-66.
9. Glimm, E., Heuer, H., Engelen, B., Smalla, K., and Backhaus, H., 1997. Statistical comparisons of community catabolic profiles. J. Microbiol. Methods 30:71-80.
10. Greaves, M. P., and Malkomes, M. P. 1980. Effects on soil microflora. Pages 223-253 in: Interactions Between Herbicides and the Soil. R. J. Hance, ed. Academic Press, New York.
11. Harikrishnan, R., and Yang, X. B. 2002. Effects of herbicides on root rot and damping-off caused by *Rhizoctonia solani* in glyphosate-tolerant soybean. Plant Dis. 86:1369-1373.
12. Hoy, J. W., and Schneider, R. W. 1988. Role of *Pythium* in sugarcane stubble decline: Pathogenicity and virulence of *Pythium* species. Phytopathology 78:1688-1692.
13. Keen, N. T., Holliday, M. J., and Yoshikawa, M. 1982. Effects of glyphosate on glyceollin production and the expression of resistance to *Phytophthora megasperma* f. sp. *glycinea* in soybean. Phytopathology 72:1467-1470.
14. Landini, S., Graham, M. Y., and Graham, T. L. 2003. Lactofen induces isoflavin accumulation and gyceollin elicitation competency in soybean. Phytochemistry 62:865-874.
15. Larkin, R. P. 2002. Effects of different 3-yr cropping systems on Rhizoctonia canker and black scurf of potato in northern Maine, 2000 and 2001. Biol. Cult. Tests Control Plant Dis. 17:PT07.
16. Larkin, R. P. 2003. Characterization of soil microbial communities under different potato cropping systems by microbial population dynamics, substrate utilization, and fatty acid profiles. Soil Biol. Biochem. 35:1451-1466.
17. Lee, Y. S., and Hoy, J. W. 1992. Interactions among *Pythium* species affecting root rot of sugarcane. Plant Dis. 76:735-739.
18. Levene, B. C., Owen, M. D. K., and Tylka, G. L. 1998. Response of soybean cyst nematodes and soybeans (*Glycine max*) to herbicides. Weed Sci. 46:264-270.
19. Lupwayi, N. Z., Rice, W. A., and Clayton, G. W. 1998. Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. Soil Biol. Biochem. 30:1733-1741.
20. Magarey, R. C. 1996. Microbial aspects of sugarcane yield decline. Aust. J. Agric. Res. 47:307-322.
21. Magarey, R. C. 1999. Reduced productivity in long-term monoculture: Where are we placed? Aust. Plant Pathol. 28:11-20.
22. Nelson, K. A., Renner, K. A., and Hammerschmidt, R. 2002. Cultivar and herbicide selection affects soybean development and the incidence of Sclerotinia stem rot. Agron. J. 94:1270-1281.
23. Nelson, K. A., Renner, K. A., and Hammerschmidt, R. 2002. Effects of protoporhyrinogen oxidase inhibitors on soybean (*Glycine max* L.) response, *Sclerotinia sclerotiorum* disease development, and phytoalexin production by soybean. Weed Technol. 16:353-359.
24. Pankhurst, C. E., Hawke, B. G., Holt, J. A., Magarey, R. C., and Garside, A. L. 2000. Effect of rotation breaks on the diversity of bacteria in the rhizosphere of sugarcane and its potential impact on yield decline. Proc. Aust. Soc. Sugar Cane Technol. 22:77-83.
25. Rands, R. D., and Dopp, E. 1938. Pythium root rot of sugarcane. U.S. Dep. Agric. Tech. Bull. 666.
26. Simon-Sylvestre, G., and Fournier, J. C. 1979. Effects of pesticides on the soil microflora. Adv. Agron. 31:1-92.
27. Smalla, K. 1998. Analysis of Biolog GN substrate utilization patterns by microbial communities. Appl. Environ. Microbiol. 64:1220-1225.
28. Staddon, W. J., Duchesne, L. C., and Trevors, J. T. 1997. Microbial diversity and community structure of postdisturbance forest soils as determined by sole-carbon-source utilization patterns. Microbial Ecol. 34:125-130.
29. Van der Plaats-Niterink, A. J. 1981. Monograph of the genus *Pythium*. Stud. Mycol. 21:1-242.
30. Viator, B. J., Griffin, J. L., and Ellis, J. M. 2002. Red morningglory (*Ipomoea coccinea*) control with sulfentrazone and azafeniden applied at layby in sugarcane (*Saccharum* spp.). Weed Technol. 16:142-148.

European Journal of Plant Pathology **109:** 445–458, 2003.

Aerial contamination of sugarcane in Guadeloupe by two strains of *Xanthomonas albilineans*

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Accepted 18 January 2003

Key words: leaf scald, epidemiology, *Saccharum* spp., virulence, epiphytic, phyllosphere, leaf surface

Abstract

Two sugarcane plots were set up in Guadeloupe with disease-free tissue cultured plants in a banana growing location distant from sugarcane fields. Thirteen weeks after planting sugarcane in the field, a *Xanthomonas albilineans* strain belonging to serotype 3 (strain XaS3) was detected in water sampled at sunrise on the leaves in the first plot. This strain randomly invaded the sugarcane canopy. Seven weeks later, a new strain belonging to serotype 1 (strain XaS1) appeared on leaves and populations of strain XaS1 progressively increased on the leaf surface, whereas populations of strain XaS3 progressively decreased. Leaf scald symptoms were first noted 26 weeks after sugarcane planting. However, only strain XaS1 was isolated from leaves and a few sugarcane stalks showing symptoms. Both strains also colonized the second field plot, which was studied at the end of the experiment to avoid human interference of aerial contamination of sugarcane. After inoculation of three sugarcane cultivars by the decapitation technique, strain XaS1 was as virulent or more virulent than five other strains of *X. albilineans* isolated from diseased sugarcane plants in Guadeloupe. Although strain XaS3 colonized a few stalks, it failed to produce any symptoms and was the least virulent strain. Leaf surface colonization by *X. albilineans* was reproduced in a greenhouse trial by spraying the pathogen on sugarcane foliage. After 8 weeks, the pathogen was isolated from disinfected leaf blades. Although the leaf scald pathogen is thought to be mainly transmitted by infected cuttings, aerial transmission of *X. albilineans* is also known to occur. These results indicate the importance of sugarcane phyllosphere colonization by virulent strains in the epidemiological cycle of leaf scald disease in Guadeloupe.

Introduction

Leaf scald, caused by *Xanthomonas albilineans*, is one of the major diseases of sugarcane (*Saccharum* spp.) and occurs in at least 66 countries of the world (Rott and Davis, 2000). It is a vascular bacterial disease that can cause severe yield losses (Martin and Robinson, 1961). Numerous haplotypes and at least three serotypes of the pathogen occur (Davis et al., 1997; Rott et al., 1994b). Leaf scald is managed by planting healthy seed-cane from plants issued from disease-free tissue culture propagation (Feldmann et al., 1994; Flynn and Anderlini, 1990) or from hot-water treated plants (Egan and Sturgess, 1980), and by use of resistant cultivars (Walker, 1971). Management of the disease is complicated by latent infection. Stalks can be infected by the pathogen for several months without showing symptoms. Improvement of diagnostic techniques has allowed detection of the pathogen in numerous symptomless plants (Comstock and Irey, 1992) revealing that *X. albilineans* seems to be randomly distributed among stalk internodes (Pan et al., 1999). Symptomless sugarcane plants can therefore constitute inoculum sources for contamination of the crop. Additionally, various epidemiological factors play a role in field contamination. Leaf scald is transmitted mechanically by

knives and harvesters and by planting infected setts (Ricaud and Ryan, 1989). However, other means of dissemination have been suspected. The pathogen was found in the rhizosphere of infected roots suggesting a possible transmission by root contact (Klett and Rott, 1994). *Xanthomonas albilineans* was also found in guttation droplets (Sordi and Tokeshi, 1986; Autrey et al., 1995), on the leaf surface of symptomatic and symptomless plants, and in aerosols above a diseased field (Klett and Rott, 1994). Leaf scald in Mauritius spread from sugarcane to maize grown between sugarcane rows (Autrey et al., 1995). These data support aerial contamination of sugarcane plants next to contaminated stalks. Leaf scald outbreaks in Florida, Louisiana and Texas were suspected to be linked to strains of *X. albilineans* that survive better epiphytically or have a greater propensity toward aerial transmission (Davis et al., 1997). In Guadeloupe, symptoms that were attributed to aerial transmission of leaf scald were first observed in sugarcane fields in 1993. These symptoms were 3–20 cm elongated necrotic stripes, generally originating from the leaf margin, from which a yellowish stripe ran down the leaf parallel to the main vein. The necrotic stripes were 0.5–1 cm wide, and the chlorotic stripes were 0.2–0.4 cm wide. *Xanthomonas albilineans* was isolated from necrotic leaves but not from the stalks. Similar symptoms were described in Mauritius in 1995 (Autrey et al., 1995) and in Florida in 2001 (Comstock, 2001). Since 1993, the presence of symptoms attributed to aerial contamination was checked each year, in Guadeloupe, in nursery plots established with disease-free material propagated by tissue culture. In this Caribbean island, 1993 was followed by 2 years of drought, and no leaf scald symptoms were observed in nurseries during that period. With the return of normal rainfall in 1996, elongated necrotic leaf symptoms attributed to leaf scald were observed in a nursery plot established with disease-free tissue cultured plants of cultivar B69566 that is susceptible to leaf scald (Rott et al., 1995). *Xanthomonas albilineans* was isolated from 18 out of 20 symptomatic leaf samples, but the pathogen was not isolated from the stalk internodes surrounded by symptomatic leaves. However, 3 months later, *X. albilineans* was isolated from 6 out of 108 (5%) stalks sampled from plants with leaf symptoms attributed to aerial transmission of leaf scald (J.H. Daugrois and L. Costet, unpublished data). These results were the first evidence of sugarcane infection in a disease-free established sugarcane field in the Carribean, by the leaf scald pathogen after aerial transmission. Similar observation was reported from Mauritius (Saumtally et al., 1996). The aim of the present study was to investigate establishment and progress of aerial transmission of *X. albilineans* on three sugarcane cultivars in disease-free fields and to determine the associated plant colonization process. Additionally, virulence of epiphytic *X. albilineans* isolates was compared with virulence of isolates previously sampled from diseased plants in Guadeloupe.

Materials and methods

Plant material

All experiments were conducted with disease-free tissue cultured plantlets. Plants were propagated *in vitro* and transferred to the greenhouse (Feldmann et al., 1994). Four-week-old greenhouse plants were used for field and greenhouse experiments.

Bacterial strains

Seven strains of *X. albilineans* isolated in Guadeloupe were used for virulence tests in greenhouse experiments (Table 1). Bacteria were stored in sterile distilled water at −20 °C until inoculation assays were performed. They were grown on Wilbrink's medium (Dye, 1980) and propagated in susceptible cultivar CP68-1026. Cultivar CP68-1026 was inoculated with a 10^8 cfu ml^{-1} pathogen suspension by the decapitation technique (Rott et al., 1997). Strains of *X. albilineans* were isolated from symptomatic leaves, subcultured and grown for 48 h on Wilbrink's medium and used to inoculate the sugarcane plants. Material used for plant inoculation was disinfected with 95% ethanol and flamed between each strain.

Table 1. Xanthomonas albilineans strains used in the greenhouse experiments

Strain	Date of isolation	Sampling location in Guadeloupe	Serotype[1]
GPE5SR	1988	Grande Terre	1
GPE27	1991	Basse Terre	1
GPE30	1991	Marie Galante	1
GPE34	1994	Basse Terre	1
GPE42	1998	Grande Terre	1
XaS1	1997	Basse Terre	1
XaS3	1997	Basse Terre	3

[1]Serotypes according to Rott et al. (1994b).

Aerial transmission of X. albilineans *(field experiment)*

Two trials of 350 m^2, separated by 15 m, were established on 22 May 1997 at Capesterre, in a highly humid location of Guadeloupe. The trials were surrounded by banana fields and were 3 km from sugarcane fields. Three sugarcane cultivars with different resistance levels to leaf scald were used: B8008 (resistant), B69566 (susceptible) and CP68-1026 (highly susceptible) (Rott et al., 1995; 1997). Each trial consisted of 10 rows of 22 m of sugarcane. Two plants of B8008 and two plants of B69566 were alternately planted on the row, and each row consisted of 36 plants. In the first trial, 14 plant pairs (two consecutive plants on the row) of B8008 and 14 plant pairs of B69566 were randomly replaced by 28 plant pairs of CP68-1026. Both trials were divided into 45 quadrats of 3 m × 2.6 m comprising eight plants each (four plants on each of the two neighbour rows). Cultivar CP68-1026 was represented in the first trial in 28 quadrats. Plants were grown according to standard commercial practices (Rott et al., 1995).

During plant growth, the dew and/or rain water available on leaves early in the morning was used for detection of *X. albilineans*. Until the first detection of *X. albilineans*, 2 ml of pooled water droplets were sampled from the leaf surface of sugarcane plants. Droplets were taken from 10 quadrats randomly chosen in each trial, and sampling was performed every 3 weeks. As soon as *X. albilineans* was detected for the first time in the field, the sampling procedure was modified and eight droplets (total volume of 80–200 µl) were sampled from each quadrat and pooled in 2 ml of sterile, distilled water. Each pooled sample and its 100-fold dilution in sterile distilled water were plated on XAS medium, modified Wilbrink's medium supplemented with KBr 5 $g\,l^{-1}$, benomyl 2 $mg\,l^{-1}$, cycloheximide 100 $mg\,l^{-1}$, propiconazole 10 $mg\,l^{-1}$, cephalexin 25 $mg\,l^{-1}$, novobiocin 30 $mg\,l^{-1}$ and kasugamycin 50 $mg\,l^{-1}$ (Davis et al., 1994), using the Spiral system (Interscience, 78 860 St Non-La-Breteche, France) to determine population size of *X. albilineans*. Bacterial populations were recorded as log[(cfu/droplet) + 1] for each sample of eight droplets in 2 ml of water. Sampling was undertaken 6, 9, 10, 13, 16, 17, 18, 20, 23, 24 and 28 weeks after planting for the first trial and 6, 9, 10, 13, 16 and 26 weeks after planting for the second trial. Necrotic leaf symptoms attributed to *X. albilineans* (Autrey et al., 1995; Comstock, 2001) were recorded on 28 November 1997 (27 weeks after planting) for each plant-pair in both trials and on 9 December 1997 (29 weeks after planting) for each stalk in the first trial. Symptoms were recorded according to a 0–3 scale: 0: no symptom, 1: one short (1–10 cm) necrotic lesion per stalk, 2: several short necrotic lesions or one large (>15 cm) necrotic lesion per stalk, 3: two or more large necrotic lesions per stalk.

Isolation of the pathogen from leaf lesions and symptomless leaf tissue was attempted after disinfecting the leaf surface with 95% ethanol. Surface-disinfected and air-dried leaf tissues were cut into small pieces in 2 ml sterile, distilled water and, 40 min later, 50 µl of the suspension was streaked on XAS medium. Isolation of the pathogen from stalks was performed with 38-week-old plants. A total of 240 stalks were sampled. Stalk colonization by *X. albilineans* was assessed by the stalk blot isolation technique with XAS medium (Davis et al., 1994). Visual identification of *X. albilineans* was randomly verified by serology (Rott et al., 1994b).

Epiphytic survival of X. albilineans *(greenhouse experiment)*

Experiment setting. Ninety-six 4-week-old plants of sugarcane cultivar CP68-1026 were transplanted into 4 l pots containing an equal volume of field soil and crushed volcanic rock and divided into 12 blocks. Plants were automatically fertilized by sprinkling irrigation with water containing 16 $mg\,l^{-1}$ of Mairol OR fertilizer (Mairol Gmbh & Co., 89547 Gussenstadt, Germany). After 3 months of growth, plants were sprayed with 5.5 l of a 10^8 cfu ml^{-1} suspension of *X. albilineans* strain GPE5SR resistant to streptomycin and rifampicin (Rott et al., 1994b). This inoculation was performed during late afternoon after the last irrigation. Four Petri dishes containing Wilbrink's medium amended with 50 $mg\,l^{-1}$ streptomycin and 50 $mg\,l^{-1}$ rifampicin (WSR medium) (Rott et al., 1994a) were placed between pots at floor level. Two days after inoculation, a bacterial film was visible on the Petri dishes.

Determination of X. albilineans *populations on plants during plant growth.* Epiphytic *X. albilineans* population densities were estimated in each block immediately and 1, 3, 7, 10, 14, 17, 24, 28 and 38 days after inoculation. Two different sampling procedures were performed. The first procedure consisted of washing two leaf parts per block with sterilized distilled

water. Six centimetre-cross sections from the middle of leaves ranked L + 2 (= second fully emerged leaf from the top) at inoculation were dipped for 1 min in a Petri dish containing 10 ml of water. During immersion of the leaves, 5 ml of water was pipetted several times and gently released along the leaf surface. The second method consisted of sampling 1 ml of water available between the stalk and the sheath of the L + 2 leaf. One water sample was randomly taken per block.

Determination of X. albilineans *populations on and in plants at the end of the experiment.* Eight weeks after inoculation, the first destructive sampling was undertaken with two stalks per block. For each stalk, the leaf blades and leaf sheaths were removed, separated and then separately washed by dipping in 300 ml of sterile, distilled water in a 0.5 l graduated cylinder and hand shaken for 1 min. The cut end of leaf blades and sheaths was kept outside the graduated cylinder sealed with cling film. All leaf blades or leaf sheaths from one plant were then homogenized for 10 min in a Warring blender in 300 ml of sterile distilled water. Sap also was extracted from a 2-cm section from each stalk internode by centrifugation at 1500*g* for 20 min. The second destructive sampling was performed 9 weeks after inoculation, and one stalk was taken in each of 5 (set 1) and 10 (set 2) randomly chosen blocks. The position (rank) of each leaf on each stalk was identified, and each leaf was treated separately. The pathogen was isolated from the leaf blade of set 1 by cutting each leaf into small pieces in 100 ml of Tris buffer saline (TBS, pH 7.5, 5 mM). Samples were shaken for 2 h on a rotary shaker before plating on WSR medium with the spiral system. The pathogen was isolated from the leaf blade of set 2 as described for those of set 1 except that leaves were previously washed and disinfected as follow. Each leaf was washed with 100 ml of 5 mM TBS by hand agitation, for 1 min, in a 250 ml graduated cylinder. It was then cut transversly into two equal parts. Both parts were then cut longitudinally and one half part of the upper part and one of the lower part were disinfected by dipping into 95% alcohol for 3 s and removing the excess alcohol by evaporation under a laminar flow cabinet. The two other half parts were disinfected by dipping into 95% alcohol for 3 s and rapid flaming. The four samples of each leaf blade of set 2 were then cut as described above. Pathogen population densities were estimated by plating 50 µl of suspension on WSR medium with the Spiral system.

Virulence tests

Two separate greenhouse experiments were conducted. In both experiments, 4-week-old plants were transplanted into 4 l pots containing an equal volume of field soil and volcanic rock. Plants were fertilized and drip-irrigated every day with 2.4 l of water containing 16 mg l^{-1} of Mairol OR fertilizer. Three-month-old plants of three sugarcane cultivars differing in resistance to leaf scald disease were used. Cultivars B69379 (susceptible), B8008 (resistant) and R570 (tolerant) were inoculated with seven strains of *X. albilineans* isolated in Guadeloupe (Table 1). Strain virulence was tested using a split plot design with three (first experiment) or four (second experiment) replications, and with the cultivars as main plots. Each treatment per block consisted of three (first experiment) or two (second experiment) plants inoculated with a strain of *X. albilineans* or distilled water (control). Plants were inoculated by the decapitation technique as previously described (Rott et al., 1997). Briefly, sugarcane stalks were cut at the third visible leaf ligule from the top with pruning shears dipped in a 10^8 cfu ml^{-1} pathogen suspension. One millilitre of the pathogen suspension was deposited with a pipette on the cut stalk.

Symptoms were recorded every 2 weeks until the end of the experiment, 12 weeks after inoculation (Rott et al., 1997). All inoculated stalks were rated individually, using a symptom severity scale ranging from 0 to 5 (0 = no symptoms to 5 = death of plant or stalk with side shoots). Ratings were used to calculate mean disease severity (DS) varying between 0 and 100 per plot. $DS = 100 \sum \text{rating}/(5T)$ where T = total number of stalks. DS recorded on newly developed, non-inoculated leaves 6, 8, 10 and 12 weeks after inoculation were used for the calculation of the area under disease progress curve (AUDPC) (Campbell and Madden, 1990) for each strain of *X. albilineans*. Three months after inoculation, two cross sections of 2 cm each were sampled from each stalk with disinfected pruning shears. The first was taken from the bottom part of the stalk from the second internode below the inoculated area that showed reduced internodes. The second was taken from the upper part of the stalk from the second internode above the inoculated area. Stalk samples were then centrifuged at 1500*g* for 20 min. The resulting sap was used to determine bacterial population densities by plating 50 µl of 10- and 1000-fold dilutions of sap extract on XAS medium with the Spiral System.

Statistical analysis

SAS computer programs 6.2 (SAS Institute Inc., Cary, NC) were used for data analysis with the exception of strength of aggregation among quadrats in field trials that was examined by spatial autocorrelation analysis using the LCOR2 software (Gottwald et al., 1992b). AUDPC of each replication of each cultivar/strain combination of the virulence tests was calculated with the data from all observation times, and compared by analysis of variance. Stalk populations of *X. albilineans* were compared by analysis of variance after rank-transformation of population density data (Rott et al., 1997).

Results

Aerial transmission to sugarcane

The first observation of *X. albilineans*-like bacteria in water droplets on leaves was made in trial one, 13 weeks after transplanting disease-free plants to the field. Priority was given to *X. albilineans* population counts in this trial and the second trial was left for sampling at the end of the experiment to observe aerial transmission of *X. albilineans* without multiple interventions during the process.

Bacteria isolated from leaf water droplets 13 weeks after transplanting, reacted positively with *X. albilineans* serotype 3 antibodies. Hereafter, this type of bacteria will be referred to as XaS3. Following first identification of XaS3 in trial one, positive quadrats were found randomly dispersed in the field during all subsequent sampling periods. No aggregated distribution was detected, and no evidence to determine entry direction of the pathogen into the field was found. The number of XaS3-positive quadrats varied from 40% to 60% until 18 weeks after planting of sugarcane in the field. Mean population densities of XaS3 rapidly increased on leaves and reached 24 cfu/water droplet 18 weeks after planting of sugarcane in the field (Figure 1). This value decreased progressively and only 2.6 cfu/water droplet were found on the leaves 28 weeks after sugarcane planting. This population density decrease was correlated with the reduction of the number of XaS3-positive quadrats. XaS3 was detected in only 14 out of 45 (31%) quadrats 24 and 28 weeks after sugarcane planting, whereas it was isolated before from 26 and 25 out of 45 (57% and 55%) quadrats, 17 and 23 weeks after sugarcane planting (Figure 2). When XaS3 densities started to decrease, a second type of *X. albilineans* strain was identified in the sampled water droplets. This new type of *X. albilineans* grew slower than XaS3 on XAS medium. After 6 days of growth, its average colony size was 1 mm compared to 3 mm for XaS3. This bacterium was isolated 20 weeks after setting up the two trials and reacted positively with *X. albilineans* serotype 1 antibodies. This type of bacteria will be referred to as XaS1 in the rest of this study. Population densities of XaS1 on the leaves increased until the end of the experiment when 5.5×10^2 cfu/droplet of water were determined (Figure 1). The mean XaS1 population at the end of the study, 28 weeks after planting of sugarcane in the field, was 200 times higher than that of XaS3. The increase of the population size of XaS1 was correlated with the increase of the number of XaS1 contaminated quadrats: 47% of the quadrats were contaminated 23 weeks after planting, whereas 98% were contaminated 5 weeks later (Table 2). The increase of XaS1 population density occurred as the XaS3 population density decreased (Figure 1). In addition, aggregated distribution was detected for XaS1 at week 23, and quadrat contamination by this strain clearly began from the west and then progressively covered the entire field (Figure 3). At the end of the experiment (28 weeks after planting), population

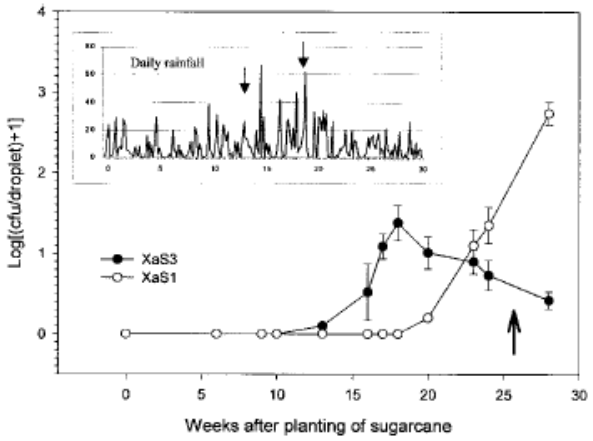

Figure 1. Xanthomonas albilineans population densities in water droplets sampled for 7 months from the leaf surface of sugarcane in the first field trial. Each vertical bar represents the standard error of the mean of 45 samples of 8 pooled droplets each. The large black arrow indicates the first observation of necrotic symptoms attributed to leaf scald. The small arrows indicate the tropical disturbances preceding strain isolation. Daily rainfall expressed in mm.

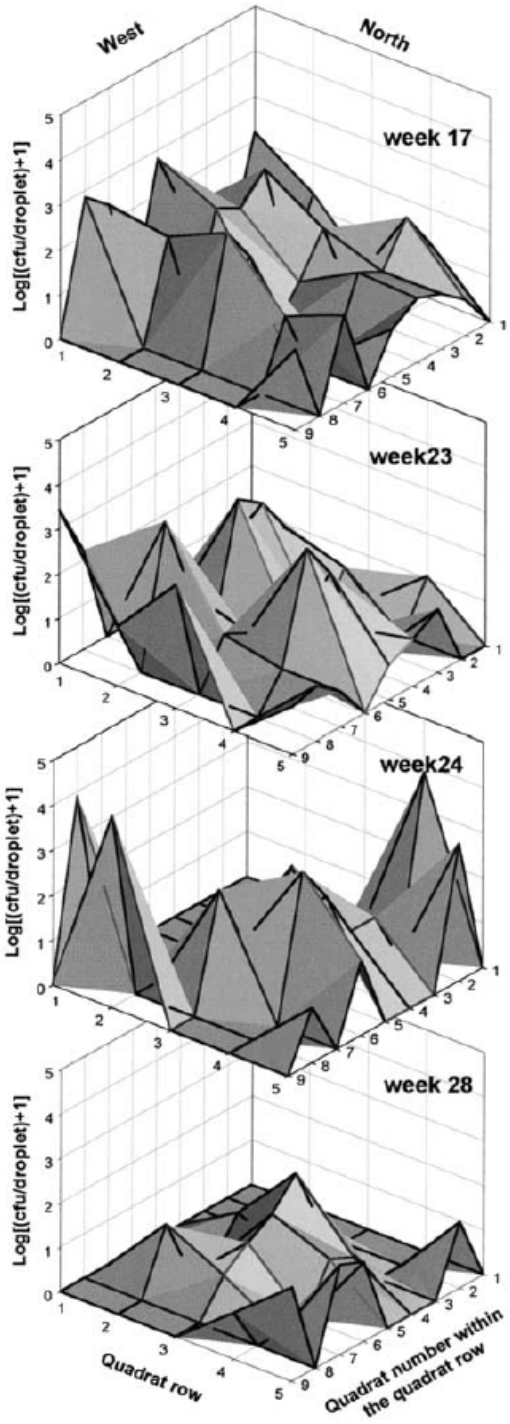

Figure 2. Distribution of *X. albilineans* serotype 3 (XaS3) in field trial one at four different dates of sampling. Pathogen population densities are expressed as the means of log 10[(cfu/droplet) + 1] per quadrat.

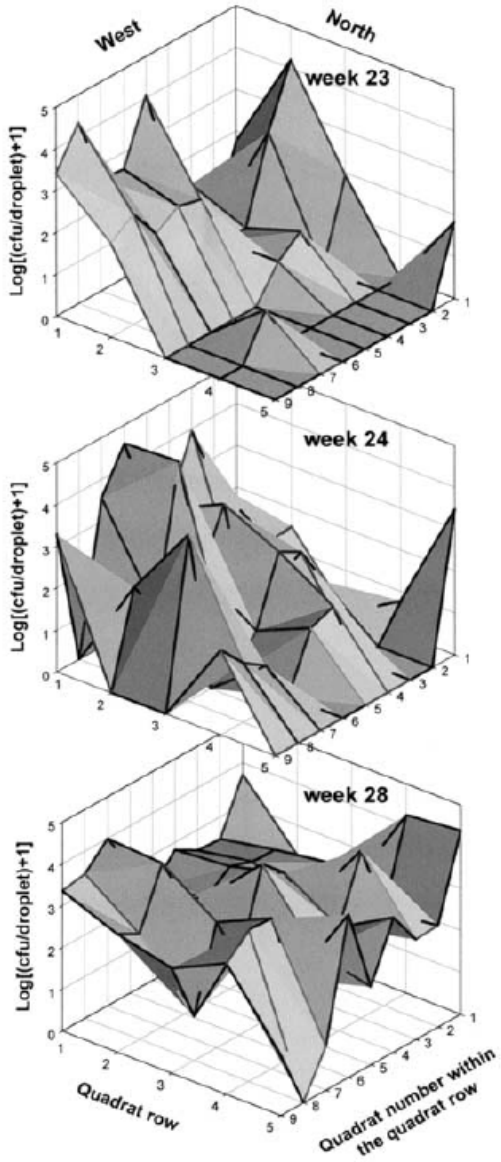

Figure 3. Distribution of *X. albilineans* serotype 1 (XaS1) in field trial one at three different dates of sampling. Pathogen population densities are expressed as the means of log 10[(cfu/droplet) + 1] per quadrat.

densities of the pathogen varied between 10 and 2.4×10^4 cfu/water droplet in each of the 44 contaminated quadrats (Figure 3). However, for 34 (77%) of the 44 contaminated quadrats, population densities varied only between 10^2 and 8×10^3 cfu/water droplet.

Similar results were obtained for trial 2 in which both serotypes were also identified (Table 2) and, as

Table 2. Leaf surface population of the leaf scald pathogen in two field trials, 5 and 6 months after planting of sugarcane in the field

Trial	Weeks after planting	Number of quadrats[1] contaminated by *X. albilineans*[2]			*X. albilineans* population densities[3]	
		XaS3	XaS1	XaS3 and XaS1	XaS3	XaS1
1	23	25 (55)	21 (47)	10 (22)	0.90	1.08
1	28	14 (31)	44 (98)	14 (31)	0.42	2.74
2	26	22 (49)	24 (53)	10 (22)	0.70	0.93

[1] Positive quadrats out of 45, in parentheses the percent corresponding value.
[2] XaS3 and XaS1 refer to serotype 3 and 1 of *X. albilineans*, respectively.
[3] Densities expressed as log[(cfu/droplet) + 1] (mean of 45 quadrats).

Figure 4. Necrotic symptoms of leaf scald on sugarcane leaves of cultivar B69566 after aerial contamination by *X. albilineans*.

in trial 1, the distribution patterns of strains XaS1 and XaS3 were different (data not shown).

Necrotic symptoms were observed on sugarcane leaves in trial one 6 weeks after the first detection of XaS1 in water droplets (Figures 1 and 4). Symptoms appeared as elongated necrotic lesions which extended along leaf veins with yellowish stripes. Thirty out of 180 plant-pairs (17%) showed these lesions 27 weeks after planting sugarcane in the field. A second observation made on each stalk showed that all three sugarcane cultivars had some necrotic leaf symptoms, but the percentage of symptomatic stalks and symptom severity varied according to cultivar (Table 3). Lesions were more frequent and more severe on cultivar B69566 than on the two other cultivars, including cultivar CP68-1026 which is highly susceptible to leaf scald. Most symptoms were recorded in the west part of the field (Figure 5) where high populations of XaS1 were found 6 weeks earlier (Figure 3). The coefficient of determination (r^2) between symptom severity observed at

week 29 and XaS1 bacterial populations in the water droplets collected on the leaves at week 23, 24 and 28 was 0.71, 0.52 and 0.24, respectively. Necrotic symptoms were also observed on few sugarcane leaves in trial two 27 weeks after planting. Samples were taken in trial one from symptomatic and asymptomatic leaves to isolate the pathogen from internal leaf tissues. XaS1 was recovered from all the 20 samples taken from the area surrounding lesions. It was also isolated from one sample out of 20 asymptomatic leaves. XaS3 was never recovered from the 40 leaf samples. Stalk infection by *X. albilineans* was investigated at the end of trial one, and XaS1 was found by stalk blot isolation with XAS medium in the lower part of two stalks out of 80 of cultivar B69566 (susceptible) and two stalks out of 72 of cultivar CP68-1026 (highly susceptible). It was also isolated from the upper part of one stalk out of 80 of cultivar B8008 (resistant).

Table 3. Necrotic leaf lesions observed on three sugarcane cultivars 29 weeks after planting (trial one, 9 weeks after first detection of *X. albilineans* XaS1 in water droplets)

Cultivar	Number of stalks observed	Percentage of stalks with score[1]			
		0	1	2	3
B69566 (S)[2]	1463	82.9	8.8	5	3.3
B8008 (R)	1290	96.1	2.6	0.9	0.4
CP68-1026 (HS)	212	94.8	3.3	1.9	0

[1] Score: 0 = no symptom, 1 = one short (1–10 cm) necrotic lesion per stalk, 2 = several short necrotic lesions or one large (>15 cm) necrotic lesion per stalk, 3 = two or more large necrotic lesions per stalk.
[2] S = susceptible to leaf scald, R = resistant, HS = highly susceptible.

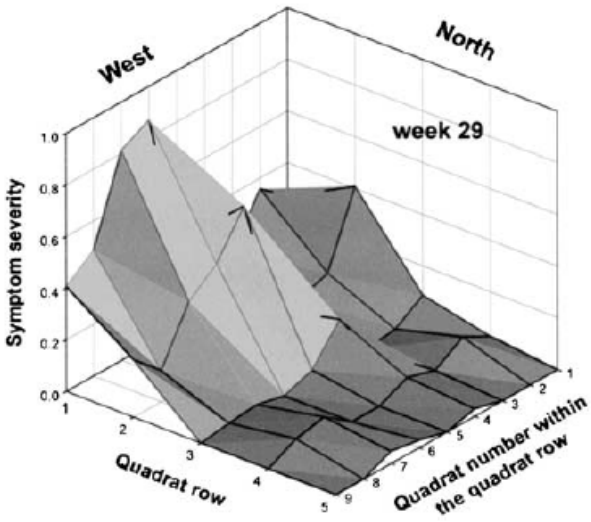

Figure 5. Symptom severity per quadrat in field trial one. Symptoms were recorded on week 29 on each stalk according to a 0–3 scale. Score: 0 = no symptom, 1 = one short (1–10 cm) necrotic lesion per stalk, 2 = several short necrotic lesions or one large (>15 cm) necrotic lesion per stalk, 3 = two or more large necrotic lesions per stalk. Data represent the mean of rating per quadrat.

Epiphytic survival of X. albilineans *(Greenhouse experiment)*

When sugarcane foliage was sprayed with *X. albilineans* strain GPE5SR in the greenhouse, mean pathogen populations isolated by leaf washing rapidly decreased on the second fully emerged leaf from the top (Figure 6). From then on, only a few bacteria (20–80 cfu ml^{-1} water) were sporadically found in some blocks until 28 days after inoculation. GPE5SR also was found in water available between the stalk and the sheath of the same leaf during 3 weeks after foliage inoculation (Figure 6). Eight weeks after inoculation, *X. albilineans* was isolated by leaf washing and leaf blade homogenization following leaf blade washing (Table 4). The pathogen was also isolated from leaf sheaths by washing and by homogenization but occurrence of positive samples was lower (Table 4).

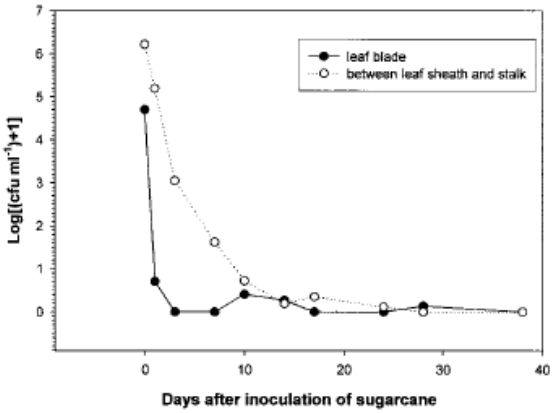

Figure 6. Xanthomonas albilineans populations on sugarcane leaf L+2 (= second fully emerged leaf from the top) and between stalk and L + 2 leaf sheath; counts were made for 38 days after inoculation of cultivar CP68-1026 by spraying the foliage with a 10^8 cfu ml^{-1} suspension of strain GPE5SR (approx. 55 ml per plant) (greenhouse trial).

Table 4. Isolation of *X. albilineans* (Xa) strain GPE5SR from CP68-1026 plants 8 weeks after spray inoculation of foliage in a greenhouse experiment

	Isolation methods				
	Leaf blade[1] wash	Leaf blade[1] homogenization	Leaf sheath[1] wash	Leaf sheath[1] homogenization	Stalk tissue[2] centrifugation
Number of positive samples/total samples	15/24	20/24	6/24	4/24	0/144
Mean Xa population log[(cfu/plant) + 1]	3.1	6.4	1.1	0.7	0

[1] Pooled leaf tissue of each plant.
[2] Six internodes per stalk taken from 24 stalks.

Table 5. Isolation of *X. albilineans* strain GPE5SR (Xa) on and in leaves of sugarcane cultivar CP68-1026, 9 weeks after spray inoculation of the foliage in a greenhouse experiment

Foliar ranks[3]	Set 1[1]		Set 2[2]					
	Positive leaf blades/total	Xa log[(cfu/leaf)+1]	Washing		Alcohol		Alcohol and flaming	
			Positive[4] leaf blades/total	Entire leaf blade log[(cfu/leaf) + 1][5]	Upper part log[(cfu/leaf) + 1][5]	Lower part log[(cfu/leaf) + 1][5]	Upper part log[(cfu/leaf) + 1][5]	Lower part log[(cfu/leaf) + 1][5]
L0	0/5	0						
L + 1	0/5	0						
L + 2	0/5	0						
L + 3	0/5	0						
L + 4	1/5	1.1	0/10	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
L + 5	2/5	2.8	3/9	0.6 (1)	3.0 (3)	0.0 (0)	0.8 (1)	0.0 (0)
L + 6	2/5	3.1	5/8	1.7 (2)	5.8 (5)	2.3 (2)	1.1 (1)	0.0 (0)
L + 7	2/5	3.7	2/4	0.0 (0)	2.3 (1)	0.0 (0)	2.1 (1)	0.0 (0)
L + 8	4/4	8.3	4/4	7.4 (4)	9.4 (4)	4.3 (2)	2.1 (1)	1.5 (1)

[1] 5 stalks were sampled, *X. albilineans* was isolated by leaf blade cutting without washing or disinfection.
[2] 10 stalks were sampled, *X. albilineans* was isolated by leaf blade washing and by leaf blade cutting after leaf washing and disinfection with alcohol with and without flaming.
[3] Foliar ranks L + 4 to L + 8 refer to leaves inoculated with the pathogen and foliar ranks L0 to L + 3 refer to leaves that appeared subsequently to inoculation. L + 1 = top fully emerged leaf.
[4] By at least one isolation method.
[5] Mean of all leaves tested. Number of positive leaves are given in parentheses.

Strain GPE5SR was not detected in any of the 24 stalks whose leaves were contaminated or not by the pathogen. *Xanthomonas albilineans* GPE5SR was present only on or in older leaves, which were sprayed by the pathogen (Table 5). The oldest leaves supported the highest pathogen population densities whatever the method used for sampling. Strain GPE5SR was also recovered from leaves after leaf washing and disinfection with alcohol. However, the pathogen was recovered from fewer leaves after alcohol disinfection and flaming than from leaves after disinfection without flaming (Table 5). The pathogen was more frequently recovered from the upper part of the leaves than from the lower part.

Virulence of seven strains of X. albilineans *from Guadeloupe, including XaS1 and XaS3 strains*

Virulence of XaS1, XaS3 and five other *X. albilineans* strains isolated in Guadeloupe was tested in two greenhouse trials after inoculation of sugarcane plants by the stalk decapitation method. Strains were compared by disease severity and bacterial populations in sap

Table 6. Pathogen populations in stalks of 3 sugarcane cultivars (R570, B8008 and B69379) and disease severity after plant inoculation by the decapitation method with seven strains of *X. albilineans*

Xa strain	Experiment 1			Experiment 2		
	Disease severity (AUDPC)[1]	Populations in stalks[2]		Disease severity (AUDPC)[1]	Populations in stalks[2]	
		Upper part	Lower part		Upper part	Lower part
GPE5SR	9.7 b	0.8 c	4.4 a	3.1 bc	1.7 b	4.9 ab
GPE27	12.5 ab	0.2 c	4.6 a	3.1 bc	1.6 b	5.1 ab
GPE30	21.0 a	5.7 a	6.7 a	10.1 b	5.6 a	5.9 a
GPE34	13.8 ab	2.4 b	6.2 a	0.5 c	0.4 b	3.7 b
GPE42	12.5 ab	1.8 bc	6.0 a	4.7 bc	3.9 a	5.8 ab
XaS3	0 c	0.3 c	0.9 b	0 c	0.3 b	0.5 c
XaS1	3.7 bc	0.9 c	4.4 a	17.0 a	5.2 a	6.3 a
Variance analysis						
Source	df	Pr > *F*	Pr > *F*	df	Pr > *F*	Pr > *F*
Cultivar	2	0.332	0.003	2	0.010	0.0002
Strain	6	0.0001	0.0005	6	0.0001	0.0001
Cultivar × strain	12	0.959	0.524	12	0.0045	0.214
Block	2	0.739	0.067	3	0.289	0.215
Cultivar × block	4	0.023	0.910	6	0.470	0.442

[1] AUDPC was calculated with the data from four observation times (6, 8, 10 and 12 weeks after inoculation).
[2] Pathogen populations isolated from sap extracted from the upper and lower part of stalks are expressed as the mean of log[(cfu ml^{-1} of sap) + 1] for all three cultivars tested. Variance analysis and Newman–Keuls grouping were performed after rank transformation of population density data.
Values followed by the same letter are not significantly different at $P = 0.05$.

extracted from stalks. Strain XaS3 failed to induce leaf scald symptoms, even on inoculated leaves, in any of the three cultivars (B8008, B69379 and R570) and in either experiment. Strain XaS1 induced only a few pencil line symptoms on leaves in the first experiment, and disease severity (AUDPC) was similar or lower than those of the other strains of *X. albilineans*. In the second experiment, however, XaS1 was the most virulent strain and showed the highest AUDPC value (Table 6).

Pathogen population densities of XaS3 in stalk sap were very low in both experiments regardless of stalk location. Additionally, only 12 stalks out of 46 were infected by this strain. Population densities of XaS1 in the lower stalk location were 2.6×10^4 and 2.0×10^6 cfu ml^{-1} of sap in trials one and two, respectively. These densities were similar or higher than those of the five other strains (GPE5SR, GPE27, GPE30, GPE34 and GPE42) (Table 6). In the upper part of the stalk, XaS1 populations were low in the first experiment (8.5 cfu ml^{-1} of sap) but similar to those of other strains (GPE5SR, GPE27 and GPE42). In the second experiment, these values reached 1.6×10^5 cfu ml^{-1} of sap when disease severity was much higher. The bacterial population density in the cultivars varied, with high levels in the tolerant cultivar R570 and low levels in the resistant cultivar B8008 (data not shown). Significant cultivar × strain interaction was only observed once for *X. albilineans* population densities in the upper part of the stalk and was not repeatable. It was, therefore, not taken into consideration to show evidence for presence of *X. albilineans* races.

Discussion

Leaf scald has previously been demonstrated to be transmitted in the field by infected setts and cutting implements. Based on symptoms, sugarcane infection by aerial contamination of disease-free plants was reported in Mauritius (Saumtally et al., 1996). Sampling water droplets from the leaf surface of sugarcane allowed us to show evidence for epiphytic existence and/or aerial dissemination of *X. albilineans* prior to plant infection, even if the field is not located in a sugarcane growing area. Water sampling on leaves is not usual for epiphytic bacterial counts (Hirano and Upper, 1983), but it was chosen because it was non-destructive. The epiphytic

population densities of *X. albilineans* are certainly higher than those present in water droplet due to bacterial adherence on leaves and/or to the part of the population that reached endophyte sites. External and internal leaf associated phytopathogenic bacteria are supposed to form a continuum due to ingression and egression process (Beattie and Lindow, 1999). Bacterial population of *X. albilineans* in water droplets may, therefore, only represent a part of the epiphytic population resulting or not from the egression process, and may have a major role in plant to plant contamination. In addition, droplet sampling did not damage or wound the leaves and avoided plant infection through the sampling procedure. Populations of *X. albilineans* appeared on the leaf surface of disease-free plants in sugarcane plots that were distant from other sugarcane fields in Guadeloupe. Two populations of *X. albilineans*, differentiated by their serological characteristics (Rott et al., 1994b) and colony size on Wilbrink's medium, were identified on the sugarcane foliage in two field trials: a non-aggressive strain belonging to serotype 3 (referred to as XaS3) and an aggressive one belonging to serotype 1 (referred to as XaS1). Identity of both strains was also confirmed with biochemical characteristics (data not shown). Serotype 3 antibodies were prepared against a strain isolated in Guadeloupe in 1970, but strains belonging to the serotype 3 group had not been found in Guadeloupe since 1985 (Rott et al., 1994b). Serotype 1 is the most widespread serotype around the world and the major one among strains of the Caribbean Islands (Rott et al., 1994b). In trial one, strain XaS3 was first detected during a 2-day weather tropical disturbance. XaS1, a serotype 1 strain, was first detected 7 weeks later and 1 week after a tropical storm (named Fabian) with heavy rains (Figure 1). These meteorological events might be responsible for long distance transport of the pathogen that invaded the sugarcane leaf canopy. Cyclones and rainstorms are known to be responsible for long distance bacterial dissemination (Gagnevin and Pruvost, 2001) and spread of bacteria in the field (Bernal and Berger, 1996). *Xanthomonas albilineans* outbreak in Louisiana was also suspected to be favoured by hurricane (Hoy and Grisham, 1994). However, initial distribution and development of XaS3 and XaS1 populations were different. When first detected, XaS3 was randomly distributed in the field, and it colonized at least half of the field canopy. In contrast, XaS1 invaded both trials from the edge of each plot, and then progressively replaced the less aggressive XaS3 on the leaves. Decline of the XaS3 population was correlated with the increase of the aggressive XaS1 population of *X. albilineans*. XaS3 showed a relative good epiphytic survival but was less competitive than the virulent strain XaS1. The two *X. albilineans* strains appeared in both trials, but the pathogen population dynamics of each differed. In the second trial, colonization of the leaves by XaS1 seemed to be delayed in comparison to trial one. Bacterial populations of XaS1 and XaS3, in the second trial, 26 weeks after planting were similar to those observed at week 23 after planting in the first trial. This observation is also supported by the lower number of necrotic symptoms that appeared in the second trial. In addition, strain XaS3 appeared to be restricted to the leaf surface of sugarcane but it could be virulent on other plants from which the strain migrated in the sugarcane plots. In contrast, XaS1 was able to invade the inner space of leaves and to induce appearance of symptoms revealing ingression possibility. Increasing population densities of *X. albilineans* serotype 1 on the leaf surface may have been due to the ability of pathogenic bacteria to multiply inside the leaf in protected areas, such as substomatal cavities or hydathodes, and then move to the surface of the leaves by exudation or when water becomes available on the leaves, or by any other egression process (Rudolph, 1993; Beattie and Lindow, 1995). The good epiphytic survival of the virulent XaS1 strain seems to be favoured by this ingression–egression process.

This hypothesis is supported by results of the greenhouse experiment in which sugarcane plants of cultivar CP68-1026 were sprayed with *X. albilineans* strain GPE5SR. The bacteria were detected on the foliage by washing a 6-cm leaf area, until 4 weeks after inoculation. However, they also were detected at the end of the experiment by washing entire leaves, when washing only 6 cm leaf areas was negative. Even though no symptoms appeared on leaves 8 weeks after inoculation, *X. albilineans* was isolated from washed leaves with or without disinfection (alcohol and flaming), indicating that some cells of *X. albilineans* reached protected areas within the leaf, confirming possibility of occurrence of ingression process in the infection cycle of *X. albilineans* virulent strains. *Xanthomonas albilineans* strain GPE5SR was not able to move to non inoculated leaves as bacterial population was only recovered from basal leaves that were present when plant surface was inoculated. This may be due to strain specificity. Strain GPE5SR, an antibiotic resistant mutant, might not be well adapted

for epiphytic competition, in contrast to wild strains (Davis et al., 1997; Klett and Rott, 1994). Additionally, the wild strain pool of *X. albilineans* may contain genotypes more fit for epiphytic conditions than the selected antibiotic resistant strain (Hirano and Upper, 1993). Absence of wind and rain splash in the greenhouse condition may also be one of the reasons for non-dispersal of strain GPE5SR. Nonetheless, as in the field, *X. albilineans* was able to enter sugarcane, at least protected sites, after leaf inoculation without wounding of the leaves. The absence of symptom development was probably due to insufficient growth time in the greenhouse (plants reached the roof of the greenhouse at the end of the experiment) or the necessity to have leaf lesions to access xylem vessels, because strain GPE5SR caused symptoms in the virulence test when sugarcane plants were inoculated by the decapitation method.

Results described herein also showed that *X. albilineans* serotype 1 moved from plant to plant in the field, eventually covering the whole leaf canopy of the sugarcane plot. This short distance movement was likely due to rain splashing during the warm, wet season (July to mid December). Xanthomonads are able to move from plant to plant or leaf to leaf (Stall et al., 1993). Klett and Rott (1994) showed that *X. albilineans* is exuded from symptomatic leaves and can be isolated from aerosols. In our study, colonization of the leaf canopy upon arrival of *X. albilineans* in the field trials was probably favoured by high humid periods during the day (90–100% relative humidity), periodic rainfalls and mild to warm temperatures (day and night temperatures ranged between 23 and 29 °C). These conditions are known to favour epiphytic life of xanthomonads (Stall et al., 1993).

Necrotic leaf lesions due to *X. albilineans* serotype 1 appeared 5–6 weeks after observation of high bacterial populations in the water available on leaves at sunrise. Symptoms were more severe in field locations where the pathogenic strain XaS1 was predominant 6 weeks earlier, indicating that epiphytic growth preceded appearance of leaf symptoms. A high correlation was also observed between symptoms and pathogen population in leaf water droplets detected 6 weeks earlier ($r = 0.86$). Similar phenomena were observed on citrus and pepper with other bacterial pathogens: *Xanthomonas campestris* pv. *citrumelo* and *X. campestris* pv. *vesicatoria* populations recoverable from the leaf surface were correlated with disease incidence that occurred several weeks later (Bernal and Berger, 1996; Gottwald et al., 1992a), but this characteristic had not been described for *X. albilineans* so far. Population size of *X. translucens* pv. *translucens* on wheat seedlings 2 days after inoculation also were predictive of bacterial leaf streak severity 5 days later (Stromberg et al., 1999). Cultivar identity was not taken into consideration during our sampling and no information is available regarding cultivar response to leaf surface contamination. Necrotic symptom severity following leaf infection varied, however, between cultivars, and response to leaf infection may therefore vary according to the cultivar. Effects of plant cultivar on foliar lesion intensity were also found in Florida after natural contamination of sugarcane by *X. albilineans* (Comstock, 2001). Nevertheless, rating cultivar resistance by foliar lesion intensity after foliar infection could differ from rating resistance after artificial plant inoculation as observed herein when the highly susceptible cultivar CP68-1026 (Rott et al., 1997) showed less foliar lesion intensity than the susceptible cultivar B69566.

The leaf scald pathogen is mainly disseminated by planting stalks taken from asymptomatic infected plants, and this material is considered to be the primary source of sugarcane infection (Ricaud and Ryan, 1989). Presence of *X. albilineans* was reported on leaf surface of symptomless plants nearby infected plants (Klett and Rott, 1994; Davis et al., 1997). However, herein is demonstrated for the first time that sugarcane leaf canopy may support high densities of epiphytic populations of *X. albilineans* prior to plant infection and symptom development in the field. Regarding the possibility of leaf canopy colonization by different strains of *X. albilineans*, it will be therefore unreasonable to predict disease incidence based on external population size without knowing strain identity and virulence. Stalk infection of sugarcane following leaf contamination is most likely the next step in the leaf scald cycle, as well as a source for a new epidemic in a newly established crop. Our results raise, therefore, the question regarding the source of the inoculum that invaded the two sugarcane trials. Several tropical weeds are known to be alternative hosts of *X. albilineans* and may constitute inoculum reservoirs when diseased sugarcane is absent (Birch, 2001; Rott et al., 1988). The majority of these weeds are non-persistant alternative hosts and some of them, as *Imperata cylindrica*, may be infected for a long-term period (Ricaud and Ryan, 1989). The planting material used in our study was disease-free, and the water droplets sampled from other plants

surrounding the sugarcane plots (various weeds, banana trees) were all negative (data not shown). Therefore, it is not possible to determine herein the distance of transport of the pathogen by air, or if other vectors or alternative hosts took part in *X. albilineans* dissemination.

Another important aspect is the variability of the pathogen present on the canopy. Results showed that at least two serotypes were able to colonize the leaf surface. Based on bacterial population densities measured in sugarcane stalks after plant inoculation, XaS1 strain was at least as virulent as other *X. albilineans* serotype 1 strains previously isolated from diseased sugarcane in Guadeloupe, whatever the cultivar inoculated. Symptoms induced by XaS1 after artificial plant inoculation were erratic between the two experiments. However, leaf scald symptoms are known to be unreliable for evaluation of sugarcane resistance to the disease, and bacterial populations are more accurate (Rott et al., 1997). Strain XaS3 was the least virulent strain in both greenhouse experiments and failed to induce symptoms. Additionally, only strain XaS1 was able to penetrate the leaves and to colonize a few sugarcane stalks in the field. More than 50 haplotypes have been identified within *X. albilineans*, and several were suspected to be associated with aerial dissemination of the leaf scald pathogen (Davis et al., 1997). Our study showed that variation in epiphytic survival exists among strains of *X. albilineans*. Additional investigations are needed to determine the genetic background of this characteristic of the pathogen and to determine the factors involved in aerial transmission of sugarcane leaf scald such as inoculum source, strain adaptability and leaf surface characteristics.

Acknowledgements

We thank the tissue culture laboratory of Cirad-ca in Guadeloupe for the supplying of sugarcane tissue cultured plants. Our thanks also go to Olivier Pruvost for analyzing the spatial dispersal of bacterial populations and to Jeffrey Hoy for critical reviewing of the manuscript.

References

Autrey LJC, Saumtally S, Dookun A, Sullivan S and Dhayan S (1995) Aerial transmission of the leaf scald pathogen, *Xanthomonas albilineans*. Proceedings International Society of Sugar Cane Technologists 21: 508–526

Beattie GA and Lindow SE (1995) The secret life of foliar bacterial pathogens on leaves. Annual Review of Phytopathology 33: 145–172

Beattie GA and Lindow SE (1999) Bacterial colonization of leaves: A spectrum of strategies. Phytopathology 89: 353–359

Bernal RF and Berger RD (1996) The spread of epiphytic populations of *Xanthomonas campestris* pv. *vesicatoria* on pepper in the field. Journal of Phytopathology 114: 479–484

Birch RG (2001) *Xanthomonas albilineans* and the antipathogenesis approach to disease control. Molecular Plant Pathology 2: 1–11

Campbell CL and Madden LV (1990) Temporal analysis of epidemics. I: Description and comparison of disease progression curves. In: Introduction to Plant Disease Epidemiology (pp 161–202) John Wiley & Sons, New York

Comstock JC (2001) Foliar symptoms of sugarcane leaf scald. Sugar Journal 64: 23–32

Comstock JC and Irey MS (1992) Detection of the sugarcane leaf scald pathogen, *Xanthomonas albilineans*, using tissue blot immunoassay, ELISA, and isolation techniques. Plant Disease 76: 1033–1035

Davis MJ, Rott P, Baudin P and Dean JL (1994) Evaluation of selective media and immunoassays for detection of *Xanthomonas albilineans*, causal agent of sugarcane leaf scald disease. Plant Disease 78: 78–82

Davis MJ, Rott P, Warmuth CJ, Chatenet M and Baudin P (1997) Intraspecific genomic variation within *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. Phytopathology 87: 316–324

Dye DW (1980) *Xanthomonas*. In: Shaad NW (ed) Laboratory Guide for Identification of Plant Pathogenic Bacteria (pp 45–49) American Phytopathological Society, Minnesota

Egan BT and Sturgess OW (1980) Commercial control of leaf scald disease by thermotherapy and a clean seed program. Proceedings International Society of Sugar Cane Technologists 17: 1602–1606

Feldmann P, Sapotille J, Grédoire P and Rott P (1994) Micropropagation of sugar cane. In: Teisson C (ed) *In vitro* Culture of Tropical Plants (pp 15–17) La Librairie du Cirad, Montpellier, France

Flynn JL and Anderlini TA (1990) Disease incidence and yield performance of tissue culture generated seedcane over the crop cycle in Louisiana. Journal of American Society of Sugar Cane Technologists 10: 113

Gagnevin L and Pruvost O (2001) Epidemiology and control of mango bacterial black spot. Plant Disease 85: 928–935

Gottwald TR, Graham JH and Richie SM (1992a) Relationship of leaf surface-populations of strains of *Xanthomonas campestris* pv. *citrumelo* to development of citrus bacterial spot and persistence of disease symptoms. Phytopathology 82: 625–632

Gottwald, TR, Richie SM and Campbell CL (1992b) LCOR2 – Spatial correlation analysis software for the personal computer. Plant Disease 76: 213–215

Hirano SS and Upper CD (1983) Ecology and epidemiology of foliar bacterial plant pathogens. Annual Review of Phytopathology 21: 243–269

Hirano SS and Upper CD (1993) Dynamics, spread, and persistence of a single genotype of *Pseudomonas syringae* relative to those of its conspecifics on populations of snap bean leaflets. Applied Environmental Microbiology 59: 1082–1091

Hoy JW and Grisham MP (1994) Sugarcane leaf scald distribution, symptomatology, and effect on yield in Louisiana. Plant Disease 78: 1086–1087

Klett P and Rott P (1994) Inoculum sources for the spread of leaf scald disease of sugarcane caused by *Xanthomonas albilineans* in Guadeloupe. Journal of Phytopathology 142: 283–291

Martin JP and Robinson PE (1961) Leaf scald. In: Martin JP, Abbott EV and Hughes CG (eds) Sugar-cane Disease of the World, Vol 1 (pp 79–107) Elsevier Publishing Co., Amsterdam

Pan YB, Grisham MP, Burner DM and Wei Q (1999) Distribution of the leaf scald pathogen in infected sugarcane stalks. Journal of American Society of Sugar Cane Technologists 19: 8–15

Ricaud C and Ryan CC (1989) Leaf scald. In: Ricaud C, Ryan BT, Gillaspie AG and Hughes CG (eds) Diseases of Sugarcane (pp 39–58) Elsevier Publishing Co., Amsterdam

Rott P and Davis MJ (2000) Leaf scald. In: Rott P, Bailey RA, Comstock JC, Croft BJ and Saumtally AS (eds) A Guide to Sugarcane Disease (pp 38–44) La librairie du Cirad, Montpellier France

Rott P, Chatenet M and Baudin P (1988) L'échaudure des feuilles de la canne à sucre provoquée par *Xanthomonas albilineans* (Ashby) Dowson. II. Diagnostic et spectres d'hôtes de l'agent pathogène en Afrique Tropicale. L'Agronomie Tropicale 43: 244–251

Rott P, Abel M, Soupa D, Feldmann P and Letourmy P (1994a) Population dynamics of *Xanthomonas albilineans* in sugarcane plants as determined with an antibiotic-resistant mutant. Plant Disease 78: 241–247

Rott P, Davis MJ and Baudin P (1994b) Serological variability in *Xanthomonas albilineans*, causal agent of sugarcane leaf scald disease. Plant Pathology 43: 244–252

Rott P, Soupa D, Brunet Y, Feldmann P and Letourmy P (1995) Leaf scald (*Xanthomonas albilineans*) incidence and its effect on yield in seven sugarcane cultivars in Guadeloupe. Plant Pathology 44: 1075–1084

Rott P, Mohamed IS, Klett P, Soupa D, de Saint-Albin A, Feldmann P and Letourmy P (1997) Resistance to leaf scald disease is associated with limited colonization of sugarcane and wild relatives by *Xanthomonas albilineans*. Phytopathology 87: 1202–1213

Rudolph K (1993) Infection of the plant by *Xanthomonas*. In: Swings JG and Civerolo EL (eds) *Xanthomonas* (pp 193–263) Chapman & Hall, London

Saumtally S, Medan H and Autrey LJC (1996) Evolution of aerial infection of leaf scald caused by *Xanthomonas albilineans* (Ashby) Dowson in sugarcane. Proceedings International Society of Sugar Cane Technologists 22: 493–496

Sordi RA and Tokeshi H (1986) Presence of *Xanthomonas albilineans* in guttation droplets of sugarcane and sweet corn leaves showing leaf scald disease symptoms. Sociedade dos Técnicos Açucareiros do Brasil, 4, Jul./Aug., 60–63

Stall RE, Gottwald TR, Koizum M and Schaad NC (1993) Ecology of plant pathogenic Xanthomonads. In: Swings JG and Civerolo EL (eds) *Xanthomonas* (pp 265–299) Chapman & Hall, London

Stromberg KD, Kinkel LL and Leonard KJ (1999) Relationship between phyllosphere population sizes of *Xanthomonas translucens* pv. *translucens* and bacterial leaf streak severity on wheat seedlings. Phytopathology 89: 131–135

Walker DIT (1971) Breeding for resistance. In: Heinz JD (ed) Sugarcane Improvement Through Breeding (pp 445–502) Elsevier Science Publishers B.V., Amsterdam

High Variation in Pathogenicity of Genetically Closely Related Strains of *Xanthomonas albilineans*, the Sugarcane Leaf Scald Pathogen, in Guadeloupe

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Accepted for publication 1 May 2006.

ABSTRACT

Champoiseau, P., Daugrois, J.-H., Pieretti, I., Cociancich, S., Royer, M., and Rott, P. 2006. High variation in pathogenicity of genetically closely related strains of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen, in Guadeloupe. Phytopathology 96:1081-1091.

Pathogenicity of 75 strains of *Xanthomonas albilineans* from Guadeloupe was assessed by inoculation of sugarcane cv. B69566, which is susceptible to leaf scald, and 19 of the strains were selected as representative of the variation in pathogenicity observed based on stalk colonization. In vitro production of albicidin varied among these 19 strains, but the restriction fragment length polymorphism pattern of their albicidin biosynthesis genes was identical. Similarly, no genomic variation was found among strains by pulsed-field gel electrophoresis. Some variation among strains was found by amplified fragment length polymorphism, but no relationship between this genetic variation and variation in pathogenicity was found. Only 3 (*pilB*, *rpfA*, and *xpsE*) of 40 genes involved in pathogenicity of bacterial species closely related to *X. albilineans* could be amplified by polymerase chain reaction from total genomic DNA of all nine strains tested of *X. albilineans* differing in pathogenicity in Guadeloupe. Nucleotide sequences of these genes were 100% identical among strains, and a phylogenetic study with these genes and housekeeping genes *efp* and *ihfA* suggested that *X. albilineans* is on an evolutionary road between the *X. campestris* group and *Xylella fastidiosa*, another vascular plant pathogen. Sequencing of the complete genome of *Xanthomonas albilineans* could be the next step in deciphering molecular mechanisms involved in pathogenicity of *X. albilineans*.

Xanthomonas albilineans is a systemic, xylem-invading pathogen that causes leaf scald of sugarcane (*Saccharum* interspecific hybrids). This disease occurs in at least 66 countries in the world and can cause severe yield losses (38). Leaf symptoms vary from a single, white, narrow, sharply defined stripe to complete wilting and necrosis of infected leaves, leading to plant death in the most susceptible cultivars. A common symptom in mature cane is the abnormal development of side shoots on stalks (38). Observation of symptoms on leaves or determination of bacterial population density in sugarcane stalk generally are used to assess disease severity and resistance after artificial inoculation of sugarcane (15,17,26,40).

The sugarcane leaf scald pathogen is spread mainly in infected sugarcane cuttings (38), and colonization of the sugarcane stalk is a critical step in disease progress. Pathogenicity of *X. albilineans*, based on the capacity of the pathogen to colonize sugarcane stalks or cause symptoms, varies according to the strain of the pathogen, indicating the existence of different pathotypes within the species (12,15,28). A major toxic compound, called albicidin and specifically produced by *X. albilineans*, plays a key role in pathogenicity (4,7,8,53). Albicidin inhibits DNA replication of proplastids, thus blocking plastid development (8). Albicidin also inhibits DNA replication of *Escherichia coli* and has antibiotic activity against several gram-positive and gram-negative bacteria (5,6). Hypothetically, albicidin also confers an advantage for sugarcane stalk colonization and disease development (4). However, no relationship was found between variation in toxin biosynthesis and variation in pathogenicity of *X. albilineans* (12). Another important feature in pathogenicity of *X. albilineans* is the ability of the pathogen to colonize the sugarcane leaf surface and, subsequently, to cause disease (15). Aerial transmission of the pathogen has been shown to occur in several geographic locations in the world, and was associated with recent outbreaks of the disease (2,13,15). In Guadeloupe, two strains of *X. albilineans* differed in their capacity to colonize the sugarcane leaf surface (15).

In *X. albilineans*, high variability has been shown for many different characteristics, such as colony and cell morphology, whole-cell proteins, fatty acid methyl esters, bacteriophage reaction, and immunological properties (serovars) (37). More recently, several genetic variants of the pathogen (haplotypes) and different albicidin production groups (albivars) were identified within the species (12,17,31). Most, if not all, of the disease outbreaks that occurred in the last two decades were attributed to genetic variants of *X. albilineans* identified by restriction fragment length polymorphism with pulsed-field gel electrophoresis (RFLP-PFGE) or by repetitive-sequence-based polymerase chain reaction (repPCR) (17,18). Ten PFGE groups (A to J) were described and strains associated with new outbreaks all belonged to PFGE group B. Strains belonging to PFGE group B also were ranked in single genetic groups by amplified fragment length polymorphism (AFLP) and after hybridization of total genomic DNA of *X. albilineans* with DNA probes that harbor albicidin biosynthesis genes (12,31). However, no relationship has been found among genetic, physiological, and serological variability and variation in pathogenicity of the pathogen from different geographic locations, including Guadeloupe (12). Absence of a relationship between variation in albicidin biosynthesis genes and variation in pathogenicity strongly suggested that other virulence factors must play a key role concurrently with albicidin production.

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DOI: 10.1094/PHYTO-96-1081

Mutagenesis by random insertion of transposon Tn*5* into genomic DNA has been used successfully for detection and characterization of pathogenicity-related genes in several phytopathogenic bacteria (19,36,46). Similarly, AFLP technique was useful to identify bacterial pathogenicity islands, genes involved in pathogenic fitness, and regulators of virulence of *X. axonopodis* pv. *manihotis*, after excision, amplification, cloning, and sequence homology analysis of polymorphic bands from AFLP gels (22). Screening of large number of transposon mutants of *X. albilineans* in sugarcane plants would be tedious and consume much time and space because sugarcane is a relatively large plant and disease symptoms appear only several weeks after plant inoculation.

The objective of this study was to investigate the variability in pathogenicity of *X. albilineans* from a single location (Guadeloupe), and to identify putative pathogenicity-associated markers in this pathogen. Various molecular biology methods were investigated, such as RFLP, AFLP, and PCR amplification of known pathogenicity genes, along with physiological tests regarding factors involved in pathogenicity.

MATERIALS AND METHODS

Bacterial strains and DNA preparation. In 2003, 75 strains of *X. albilineans* were collected in Guadeloupe from different sugarcane-growing locations. Single colonies were isolated on selective agar medium (XAS) after 5 days of growth at 28°C (16). Bacterial strains were stored at –20°C as turbid water suspensions immediately after isolation and retrieved on modified Wilbrink's (MW) agar medium for 3 to 5 days at 28°C just before inoculum preparation (35). Serological characteristics of strains were determined as described by Rott et al. (39). For each strain, albicidin production was quantified in vitro by a microbiological assay as described by Champoiseau et al. (12). *X. albilineans* strain Xa23R1 from Florida (36), *X. campestris* pv. *campestris* strain CFBP 5251 (E. Vivien, *personal communication*), *X. campestris* pv. *vesicatoria* strain 91-118 (1), and *X. arboricola* pv. *pruni* strain CFBP 6653 (9) were grown on MW agar for 3 days at 28°C.

E. coli strain DH5α (Life Technologies Inc., Gaithersburg, MD) was grown on Luria-Bertani (LB) agar medium for 24 h at 37°C (42).

Total genomic DNA of bacterial strains was extracted using the Qiagen extraction DNeasy Tissue kit (Qiagen SA, Courtaboeuf, France) according to the manufacturer's recommendations, and adjusted to a final concentration of 20 to 25 ng/µl.

Pathogenicity assays. Two greenhouse experiments were set up in Guadeloupe to assess pathogenicity of *X. albilineans* strains by measuring disease severity on leaves and stalk colonization by the pathogen. The experiments were conducted with disease-free tissue-cultured plants of sugarcane cv. B69566, which is susceptible to the leaf scald bacterium (40). Plants were propagated in vitro and transferred to the greenhouse as described by Feldmann et al. (20). Four-week-old plants were grown individually in a

TABLE 1. Characteristics of *Xanthomonas albilineans* strains from Guadeloupe used in this study

Strain[a]	Origin	Sampling date	Sugarcane host cv.	Isolated from[b]	Albicidin production: GIR (mm)[c]
GPEPC1	Roujol, Petit-Bourg	January 2003	HJ5741	Leaf surface	2.3 (0.4)
GPEPC5	Roujol, Petit-Bourg	January 2003	HJ5741	Stalk	2.3 (0.2)
GPEPC7	Roujol, Petit-Bourg	January 2003	HJ5741	Stalk	2.3 (0.4)
GPEPC9	Roujol, Petit-Bourg	February 2003	B60182	Leaf symptom	2.8 (0.6)
GPEPC11	Roujol, Petit-Bourg	February 2003	B60233	Leaf symptom	3.3 (0.4)
GPEPC13	Roujol, Petit-Bourg	February 2003	B61337	Leaf symptom	2.8 (0.9)
GPEPC15	Roujol, Petit-Bourg	February 2003	B64217	Leaf symptom	1.0 (0.3)
GPEPC17	Roujol, Petit-Bourg	February 2003	B64277	Leaf symptom	2.5 (0.3)
GPEPC19	Roujol, Petit-Bourg	February 2003	B721	Leaf symptom	2.2 (0.2)
GPEPC20	Roujol, Petit-Bourg	February 2003	B711001	Leaf symptom	0.5 (0)
GPEPC21	Roujol, Petit-Bourg	February 2003	B74125	Leaf symptom	2.0 (0)
GPEPC23	Roujol, Petit-Bourg	February 2003	B73520	Leaf symptom	2.5 (0.3)
GPEPC25	Roujol, Petit-Bourg	February 2003	B67215	Leaf symptom	1.2 (0.6)
GPEPC30	Roujol, Petit-Bourg	February 2003	B73146	Leaf symptom	3.0 (0.3)
GPEPC32	Roujol, Petit-Bourg	February 2003	B75548	Leaf symptom	4.2 (0.6)
GPEPC33	Roujol, Petit-Bourg	February 2003	B7678	Leaf symptom	0.5 (0)
GPEPC34	Roujol, Petit-Bourg	February 2003	B76345	Leaf symptom	0.8 (0.2)
GPEPC36	Roujol, Petit-Bourg	February 2003	B79222	Leaf symptom	3.0 (0)
GPEPC37	Roujol, Petit-Bourg	February 2003	B78172	Leaf symptom	2.7 (0.2)
GPEPC39	Roujol, Petit-Bourg	February 2003	B83127	Leaf symptom	3.0 (0.3)
GPEPC42	Roujol, Petit-Bourg	February 2003	R84379	Leaf symptom	3.5 (0)
GPEPC44	Roujol, Petit-Bourg	February 2003	R832065	Leaf symptom	2.8 (0.2)
GPEPC46	Roujol, Petit-Bourg	February 2003	BBZ8108	Leaf symptom	2.3 (0.2)
GPEPC49	Roujol, Petit-Bourg	February 2003	Beqa	Leaf symptom	2.8 (0.6)
GPEPC50	Roujol, Petit-Bourg	February 2003	BISCUIT	Leaf symptom	0.3 (0.2)
GPEPC52	Roujol, Petit-Bourg	February 2003	BJ7938	Leaf symptom	3.3 (0.4)
GPEPC53	Roujol, Petit-Bourg	February 2003	BJ84135	Leaf symptom	0.2 (0.2)
GPEPC54	Roujol, Petit-Bourg	February 2003	BJ82119	Leaf symptom	4.0 (0)
GPEPC55	Roujol, Petit-Bourg	February 2003	BNSxSES4	Leaf symptom	0.0 (0)
GPEPC57	Roujol, Petit-Bourg	February 2003	BR6223	Leaf symptom	0.0 (0)
GPEPC59	Roujol, Petit-Bourg	February 2003	BT7716	Leaf symptom	2.2 (1.1)
GPEPC61	Roujol, Petit-Bourg	February 2003	CB4013	Leaf symptom	3.8 (0.2)
GPEPC64	Roujol, Petit-Bourg	February 2003	D61107	Leaf symptom	2.7 (0.4)
GPEPC65	Roujol, Petit-Bourg	February 2003	DB62639	Leaf symptom	4.0 (0.3)
GPEPC67	Roujol, Petit-Bourg	February 2003	EROS	Leaf symptom	2.5 (0.3)

(*continued on next page*)

[a] All strains belonged to serovar I as described by Rott et al. (39).

[b] Strains from the leaf surface were isolated from water droplets collected from the leaf surface of symptomless sugarcane plants. Strains from the stalk were isolated from stalk tissue sampled from diseased sugarcane stalks. Strains from leaf symptoms were isolated from leaves showing necrosis.

[c] Albicidin production was determined as described by Champoiseau et al. (12). Growth inhibition ring (GIR) of *Escherichia coli* values are the means of three plates for each strain of *X. albilineans*. Values in parentheses are square means.

greenhouse in 4-liter pots containing an equal volume of field soil and volcanic rock. During plant growth, secondary shoots were systematically removed to keep a single stalk per plant.

Four-month-old plants were inoculated with bacterial suspensions adjusted to 10^8 CFU/ml by the decapitation technique as described by Rott et al. (40).

In the first trial (trial A), plants were inoculated with 75 strains of *X. albilineans* (Table 1). Plants then were distributed in the greenhouse using a randomized complete block design with five replications of one plant each. The second trial (trial B) was set up 4 months after trial A, and plants were inoculated with 19 strains of *X. albilineans* that were representative of the variability in pathogenicity identified in trial A (Table 2). After inoculation, plants were distributed in the greenhouse using a randomized complete block design with four replications of four plants each. Control plants inoculated with sterile distilled water were included in both trials to evaluate any naturally occurring cross contamination (17).

Disease severity was assessed as described by Champoiseau et al. (12). Briefly, symptoms were recorded on noninoculated leaves using a symptom severity scale ranging from 0 to 5, where 0 = no symptoms, 1 = one or two pencil-line streaks, 2 = more than two pencil-line streaks, 3 = leaf chlorosis or bleaching, 4 = leaf necrosis, and 5 = death of the plant. Inoculated plants were rated individually based on the score of the leaf showing the most severe symptom. Disease severity (DS) was expressed as $DS = 100([1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4 + 5 \times N_5]/5 \times N_T)$, where N_i = number of plants with score i and N_T = total number of plants. Symptoms were recorded every 2 weeks after inoculation and for 12 weeks in trial A and trial B.

Pathogen populations in different stalk locations were determined 17 and 16 weeks after plant inoculation in trial A and trial B, respectively, by the stalk blot inoculation technique with XAS selective medium, as previously described (12). In both trials, pathogen populations were determined at the location of three shortened stalk internodes that showed reduced growth after mechanical plant inoculation (cut leaves above the growing point) (SL_0) to control efficiency of plant inoculation. These internodes were attached to the inoculated leaves and, therefore, were considered to be the entry point of the pathogen into the sugarcane stalk. In trial A, pathogen populations also were determined in every two internodes above SL_0 (SL_{+2}, SL_{+4},…,SL_{+X}, where X = the last even-numbered internode near the apex of the sugarcane stalk). In trial B, pathogen populations were determined in every internode below SL_0 (SL_{-1}, SL_{-2},…, SL_{-Y}, where Y = the first internode of the sugarcane stalk near the soil level) and in every internode above SL_0 (SL_{+1}, SL_{+2},…,SL_{+Z}, where Z = the last internode near the apex of the sugarcane stalk). Pathogen population densities were assessed in the two trials using a 0-to-4 scale, where 0 = no bacterial colony in the stalk imprint, 1 = 1 to 10 colonies in the stalk imprint, 2 = >10 colonies or confluent growth of bacteria in less than 25% of the stalk imprint, 3 = confluent growth of bacteria in 25 to 75% of the stalk imprint, and 4 = confluent growth of bacteria in >75% of the stalk imprint. Visual identification of *X. albilineans* colonies or confluent growth was verified arbitrarily by serology (39).

TABLE 1. (*Continued from preceding page*)

Strain[a]	Origin	Sampling date	Sugarcane host cv.	Isolated from[b]	Albicidin production: GIR (mm)[c]
GPEPC68	Roujol, Petit-Bourg	February 2003	F148	Leaf symptom	2.0 (0)
GPEPC71	Roujol, Petit-Bourg	February 2003	FR90771	Leaf symptom	3.2 (0.4)
GPEPC73	Roujol, Petit-Bourg	February 2003	H63-1418	Leaf symptom	0.0 (0)
GPEPC75	Roujol, Petit-Bourg	February 2003	IAC58-480	Leaf symptom	3.0 (0)
GPEPC77	Roujol, Petit-Bourg	February 2003	LF610003	Leaf symptom	3.2 (0.8)
GPEPC79	Roujol, Petit-Bourg	February 2003	M377/56	Leaf symptom	0.0 (0)
GPEPC80	Roujol, Petit-Bourg	February 2003	PR1116	Leaf symptom	3.5 (0)
GPEPC81	Roujol, Petit-Bourg	February 2003	ONO	Leaf symptom	1.3 (0.2)
GPEPC83	St. Jean, Petit-Bourg	March 2003	FR90714	Leaf symptom	…
GPEPC84	St. Jean, Petit-Bourg	March 2003	SP71-6168	Leaf symptom	3.2 (0.2)
GPEPC86	Meynard, Petit-Bourg	March 2003	B69566	Leaf symptom	2.0 (0)
GPEPC87	Meynard, Petit-Bourg	March 2003	B69566	Leaf symptom	2.7 (0.2)
GPEPC89	G. Montagne, Lamentin	March 2003	B69566	Leaf symptom	0.5 (0)
GPEPC90	P. Canal, Port-Louis	March 2003	B69566	Leaf symptom	1.7 (0.2)
GPEPC91	P. Canal, Port-Louis	March 2003	B69566	Leaf symptom	0.5 (0)
GPEPC92	P. Canal, Port-Louis	March 2003	B69566	Leaf symptom	1.5 (1.0)
GPEPC94	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	0.8 (0.4)
GPEPC101	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf surface	0.0 (0)
GPEPC103	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	2.0 (0)
GPEPC106	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	1.5 (0.3)
GPEPC109	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	2.3 (0.2)
GPEPC112	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	1.2 (0.4)
GPEPC117	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	…
GPEPC120	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	…
GPEPC125	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	0.8 (0.4)
GPEPC126	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	0.0 (0)
GPEPC127	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	2.2 (0.2)
GPEPC128	St. Sauveur, Capesterre B. Eau	March 2003	B69566	Leaf symptom	2.8 (0.2)
GPEPC131	Christophe Ouest, Goyave	March 2003	B69566	Leaf symptom	3.3 (0.2)
GPEPC133	Christophe Ouest, Goyave	March 2003	B69566	Leaf symptom	3.5 (0.7)
GPEPC135	St. Louis, Marie-Galante	March 2003	B69566	Stalk	1.0 (0.7)
GPEPC137	St. Louis, Marie-Galante	March 2003	B69566	Stalk	…
GPEPC139	St. Louis, Marie-Galante	March 2003	B69566	Leaf symptom	2.7 (0.2)
GPEPC140	St. Louis, Marie-Galante	March 2003	B69566	Stalk	0.5 (0)
GPEPC141	St. Louis, Marie-Galante	March 2003	B69566	Leaf symptom	0.5 (0)
GPEPC142	Cluny, Sainte-Rose	March 2003	B69566	Leaf symptom	4.3 (0.2)
GPEPC143	Cluny, Sainte-Rose	March 2003	B69566	Leaf symptom	4.3 (0.2)
GPEPC145	Cluny, Sainte-Rose	March 2003	B69566	Leaf symptom	0.0 (0)
GPEPC146	Cluny, Sainte-Rose	March 2003	B69566	Leaf symptom	2.3 (0.2)
GPEPC148	Roujol, Petit-Bourg	March 2003	NCo376	Stalk	0.8 (0.4)

Extent of stalk colonization (ESC) was expressed as ESC = $100([1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4]/4 \times N_T)$, where N_i = number of internodes with score i and N_T = total number of internodes.

RFLP analysis of total genomic DNA. PFGE was used for RFLP analysis of the 19 strains of *X. albilineans* selected herein (Table 2), after restriction of total genomic DNA with the rare cutting enzyme *Spe*I, as described by Davis et al. (17). Strain Xa23R1 from Florida (41) was used as the reference strain.

RFLP analysis of albicidin biosynthesis genes. Two DNA plasmids (pALB571 and pBKS/K7), which cover all three genomic regions involved in albicidin biosynthesis, were used as probes for RFLP analysis of the 19 strains of *X. albilineans* selected herein (Table 2), as described by Champoiseau et al. (12). Strain Xa23R1 from Florida was used as the reference strain.

AFLP analysis. Template DNA, enzymes for restriction, specific adaptors for ligation, and primers for selective amplification were designed based on a preliminary study by Pilet et al. (31). In all, 80 to 100 ng of DNA were digested for 2 h at 37°C (followed by 10 min at 65°C) in a 25-µl reaction mixture with 6/6.4 units of *Msp*I/*Sac*I enzyme combination (Promega Corp., Madison, WI) and ligated with specific adaptors. Double-stranded DNA adaptors were prepared by 15 min of denaturation at 75°C and overnight renaturation of the two oligonucleotides in a reaction mixture containing 1× enzyme buffer and 20 µM of each oligonucleotide. *Msp*I adaptor (5′-GACGATGAGTCCTGAA-3′ and 3′-GCTACTCAGGACTTGC-5′) and *Sac*I adaptor (5′-CTCGTAGACTGCGTACAAGCT-3′ and 3′-GCATCTGACGCATGT-5′) were ligated into restricted DNA for 2.5 h at 14°C in 50 µl of reaction mixture (25 µl of restricted DNA, 1× ligase buffer, 4.2 units of T4 DNA ligase [Promega Corp.], 2× bovine serum albumen, 50 pmol *Msp*I adaptor, and 5 pmol *Sac*I adaptor). The ligation solution was diluted 2.5-fold in water and stored at −20°C before selective amplification.

The following combinations of selective primers were used for AFLP analysis because they generated reliable and easily identifiable DNA fingerprints in preliminary experiments (data not shown): *Sac*I + G (5′-TAGACTGCGTACAAGCTCG-3′)/*Msp*I + Y (5′-CGATGAGTCCTGAACGGY-3′), where Y = A, T, G, or CT. *Sac*I selective primer was labeled with ^{33}P-ATP using the T4 kinase according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA) and used for amplification. The ligation solution (5 µl) was amplified in a 20-µl reaction mixture (1× enzyme buffer, 2 mM $MgCl_2$, 0.3 mM dNTP, 0.1 µM ^{33}P-labeled *Sac*I primer, and 1 µM *Msp*I primer) containing 1 unit of Taq DNA polymerase (Eurobio, Paris, France). PCR amplifications were performed in an automated thermal cycler with the initial cycle at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s, followed by 12 cycles with subsequent reduction of annealing temperature by 0.7°C per cycle, and 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. After amplification, 20 µl of sequencing dye (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue) was added to each reaction mixture, and the final solution was heated at 94°C for 3 min and kept warm at 70°C during loading of the electrophoresis gel. A 6-µl aliquot of each sample was separated on a 5% polyacrylamide denaturing gel containing 7.5 M urea and 0.5× Tris-borate EDTA (TBE) running buffer (Qbiogene, Illkirch, France) at 55 W for 1.8 to 2.0 h, in a 30-by-40-cm sequencing apparatus. After electrophoresis, gels were covered with Saran Wrap, transferred to Whatman 3-mm chromatography paper, dried under vacuum at 80°C for 30 min, and exposed to radiographic films for 12 to 24 h. Duplicated AFLP fingerprints were obtained for each strain

TABLE 2. Disease severity on leaves and extent of stalk colonization of sugarcane cv. B69566 after inoculation with 19 strains of *Xanthomonas albilineans* from Guadeloupe[a]

		Mean (square mean)			
		Disease severity[b]		Extent of stalk colonization[c]	
Strain		Trial A	Trial B	Trial A	Trial B
GPEPC17		0.0 (0)	0.0 (0)	0.0 (0)	12.2 (14.5)
GPEPC23		0.0 (0)	1.3 (2.5)	3.8 (6.0)	7.5 (7.6)
GPEPC52		28.0 (33.6)	52.7 (27.8)	34.1 (27.3)	63.3 (26.2)
GPEPC73		52.0 (25.6)	78.8 (6.9)	81.3 (9.4)	93.4 (7.6)
GPEPC75		16.0 (21.6)	26.3 (25.3)	51.0 (20.8)	60.8 (23.7)
GPEPC79		26.0 (31.2)	40.0 (22.5)	30.5 (17.8)	67.2 (19.4)
GPEPC80		8.0 (12.8)	14.7 (17.6)	4.0 (6.4)	48.0 (21.2)
GPEPC81		0.0 (0)	2.5 (4.4)	32.6 (28.9)	16.6 (15.6)
GPEPC83		0.0 (0)	2.5 (4.4)	54.5 (18.6)	42.8 (25.9)
GPEPC84		4.0 (6.4)	0.0 (0)	3.3 (5.3)	12.2 (10.5)
GPEPC86		0.0 (0)	0.0 (0)	0.0 (0)	18.2 (16.6)
GPEPC87		28.0 (17.6)	36.9 (26.5)	61.3 (29.1)	74.2 (16.7)
GPEPC91		0.0 (0)	28.1 (26.6)	23.3 (25.7)	56.7 (27.6)
GPEPC101		0.0 (0)	0.0 (0)	5.0 (8.0)	4.9 (5.1)
GPEPC106		0.0 (0)	0.0 (0)	6.7 (10.7)	16.8 (11.8)
GPEPC125		0.0 (0)	3.1 (5.9)	3.0 (4.8)	10.2 (10.1)
GPEPC142		8.0 (12.8)	18.8 (21.1)	39.7 (27.6)	42.3 (16.5)
GPEPC143		50.0 (24.0)	44.4 (16.3)	63.4 (5.7)	73.2 (8.7)
GPEPC145		20.0 (8.0)	43.8 (15.8)	55.8 (10.3)	69.1 (11.4)
Variance analysis					
Source					
Strain	DF	18	18	18	18
Strain	($P > F$)	<0.0001	<0.0001	<0.0001	<0.0001
Bloc	DF	4	3	4	3
Bloc	$P > F$	0.137	0.383	0.287	0.566
Strain × bloc	DF	…	54	…	54
Strain × bloc	$P > F$	…	0.544	…	0.246
Error DF		72	225	71	228

[a] The experiment was set up in a greenhouse in Guadeloupe (French West Indies). Only data of the 19 strains of the pathogen common to trial A and trial B were used for statistical analysis.
[b] Disease severity on a scale of 0 to 100 was determined 12 weeks after plant inoculation in both trials.
[c] Extent of stalk colonization on a scale of 0 to 100 was determined 17 and 16 weeks after plant inoculation in trial A and trial B, respectively.

and each selective primer combination with two different DNA preparations. Only reproducible and strong DNA bands observed in both duplications were scored for DNA analysis.

Binary scores were attributed based on the presence or absence (1 or 0, respectively) of DNA bands. Binary matrices were used to calculate similarity between paired haplotypes with Jaccard's similarity index (45). Unweighted pair-group method with arithmetic means (UPGMA) dendrograms showing cluster analysis of the distance matrix were constructed with the DARwin program (version 4.0; CIRAD-FLHOR, Montpellier, France).

PCR identification of pathogenicity-related genes. Total genomic DNA of nine strains of *X. albilineans* differing in pathogenicity in Guadeloupe (GPEPC17, GPEPC73, GPEPC75, GPEPC84, GPEPC86, GPEPC87, GPEPC101, GPEPC142, and GPEPC143) and *X. albilineans* strain Xa23R1 from Florida was used as template DNA to amplify partial pathogenicity-related genes by PCR. Eighty oligonucleotide primers were designed to amplify 40 gene fragments (Table 3). Genomic DNA (1 µl) was used as template in a 50-µl reaction mixture (1× enzyme buffer, 2 mM $MgCl_2$, 0.2 mM dNTP mix, and 0.3 µM of each primer) containing 2.5 units of Taq DNA polymerase (Eurobio). PCR amplification was performed in an automated thermocycler with the following program: 95°C for 5 min, and 30 cycles that included 94°C for 1 min, annealing temperature for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were separated by electrophoresis in a 1% agarose gel in 0.5× TBE buffer (Qbiogene), stained with ethidium bromide, and visualized by UV transillumination. ProMega-Marker Lambda Ladder 1 kb (Promega Corp.) was used as molecular weight marker.

TABLE 3. Expected sizes of DNA fragments amplified from pathogenicity-related genes (I) and housekeeping genes (II) and characteristics of the oligonucleotides used as primers for polymerase chain reaction (PCR) amplification

DNA fragment[a]		Primer sequences (5′ > 3′)[b]				
Name	Size (bp)	Forward	Reverse	From	No. or reference[c]	Protein function[d]
I.						
adh	731	GAACTGTTTCGGTAGGTGATG	CTGATAACCCACACCTGCAC	Xf9a5c, Xftem	AAF84783	Surface protein
cbas	437	GATATCGGCCATTCCGGCT	CTCTGATTGCACCARTTGCC	Xac, Xcc, Xoo	AAM42804	1,4-β-Cellobiosidase
clas	353	CGGCTCSGGCTAYTCKGGC	GCACCTGGATCGCSGCCCA	Xac, Xoo	AAM42805	Cellulase
gumB	596	CTGGGCGCTTGCAGCACC	TCCAGCATGGTGCGCAACC	Xac, Xcc, Xoo	AAA86370	Gum B
gumD	1447	GCTTTTGGCAGACTTGAGTAGC	ACGCGGTCTTCTGTCCGAGC	Xac, Xcc, Xoo	AAA86372	Gum D
gumE	616	ACTGGCAGATCGACCCCAAG	CCCATCGTGGTGTCCTGGT	Xac, Xcc, Xoo	AAA86373	Gum E
gumF	736	GGCTGGCTGCCGGTTATG	GCCAAGGCCGCAACACC	Xac, Xcc, Xoo	AAA86374	Gum F
gumH	795	CGTGTGTTCACCGATC	GGCAAGCCGCACGAA	Xac, Xcc, Xoo	AAA86376	Gum H
gumI	615	GCAGCTGACCGGCAC	GCATCGAACTCGCCTTC	Xac, Xcc, Xoo	AAA86377	Gum I
gumJ	941	GTGCAGTTCGGCGGC	GCCGTCTGGAAGATACC	Xac, Xcc, Xoo	AAA86378	Gum J
gumK	596	CGCGAGTTCGACCGCG	TCGGTGGTGTCGGACC	Xac, Xcc, Xoo	AAA86379	Gum K
gumL	608	TGTTCTGGTGGCAGCCC	GTAGTAGTCGTCGTACTTG	Xac, Xcc, Xoo	AAA86380	Gum L
gumP	792	TGCGAACGCATGGTGCGC	CCACTCCGGTTGATAACGG	Xac, Xcc, Xoo	AAM41716	Gum P
hemag1	727	CGCGCCCAACTCATCATTGC	GGTTGTCCAGGTGTTGGGTC	Xf9a5c, Xftem	AAO29937	HLS protein
hemag2	734	CACCCACACCGACCTCAGC	CGGTGGTGGTGCCGAGGC	Xf9a5c, Xftem	AAO29937	HLS protein
pect	435	ACTATGTCGAAGGCACTTAC	GCGAGGTGCCGTTGACG	Xcc, Xoo	AAM41544	Pectinesterase
hrp1	840	AGGCCCTGGAAGGTGCCCTGGA	ATCGCACTGCGTACCGCGCGCGA	Xcv	Leite et al. (27)	HrcL and HrcN proteins
hrp2	355	AATACGCTGGAACTGCTG	GGCACTATGCAATGACTG	Xcv	Leite et al. (27)	HrcN and HrpB7 proteins
pgl	662	GGCCTGGGCACCTGCGGC	CGCACGGCGATATGGCGCAT	Xac, Xcc, Xoo	AAM41545	Polygalacturonase
pilB	684	CGCCGGTGGTGAAGTTCGTC	CCGCCTTGATCGCAATCTCG	Xac, Xcc, Xoo	AAP43029	Pilus biogenesis protein B
pilD	603	TCGACCAGCATCCCGGTCTC	CTTCCTTGCCGGTGAGCTGC	Xac, Xcc, Xoo	AAP43033	Type IV pre-pilin leader peptidase
pilG	331	GCGGGTGGGGAACTCGCAG	GCTTGGTCAGATATTGCTCGG	Xac, Xcc, Xoo	AAM42197	Pilus
pilH	308	TTGATCGAGGACTCGCCCAC	TCGCGCGGCGGCTTGACG	Xac, Xcc, Xoo	AAM42196	PilH
pilJ	598	ATCAGCTCGCTCGGTGAGGG	GGGTCGCACTGGAGGTGCG	Xac, Xcc, Xoo	AAM42194	Pilus biogenesis protein
rpfA1	947	ATGAGCGATTCCTTTTCCAC	GGGAAGATGCCGCAGGTG	Xac, Xcc, Xoo	AAM41149	Aconitase
rpfA2	1140	CTGCATCGGCAACTCCGGC	CCGTGCTTGAAGTACTCCAC	Xac, Xcc, Xoo	AAM41149	Aconitase
rpfB	1129	ACAGCTTCGGCAAGACC	CCCTTCATCACCTGCGG	Xac, Xcc, Xoo	AAM41147	RpfB protein
rpfC	698	GGCAATGCGGTCAAGTTC	TCGGCGCTGAGCACCAC	Xac, Xcc, Xoo	AAM41145	RpfC protein
rpfG	1041	GATTGAACATCGTCATTGTC	CCGATGCGGTGGAGAAC	Xac, Xcc, Xoo	AAM41143	RR protein
xad	914	ACCGAAGGCACCGATGCG	TCACCCCACGCCACACTG	Xac, Xcc	AAM39974	Outer membrane protein
xpsE	570	CGTGCTGGTGGAAGGCGAAAG	AGCGCGGACTGGATCGCGAT	Xac, Xcc, Xoo	AAM39976	GSP protein E
xpsF	675	CATCAACATGGTGCGCGCGG	GTGTCGGCGGTCTTGAGCAG	Xac, Xcc, Xoo	AAM39977	GSP protein F
xpsG	291	CAGGCCGGCATGAGCTTGCT	GTATTCGATGGYGTGGCCCCA	Xac, Xcc, Xoo	AAM39978	GSP protein G
xpsI	369	TGATCGAAGTGATCGTCGCCT	TGGCSGYCACCAGCCGCAA	Xac, Xcc, Xoo	AAM39980	GSP protein I
xsaA	631	CTGGCGCGCACCGCCATTT	CGTGTTCGTCGAGGATGACG	Xac, Xcc, Xoo	AAM43343	Xylosidase/arabinosidase
xsaB	772	TGTCGGTCGGCTCGGATTTTC	TAGCTGCTGCTCCACTCGGT	Xac, Xcc	AAM43196	β-Xylosidase
xylA1	615	TCCCCGGCATCGGCAAGATC	CCATGTTGTCCTGCTCGCGC	Xac, Xcc, Xoo	AAM41049	Xylose isomerase
xylA2	482	TGAAGCGCGAGCAGGACAACA	CGCGTCCATGCCGCCGATG	Xac, Xcc, Xoo	AAM41049	Xylose isomerase
xynA	486	ACCAACTGGGTGCAGCGCGT	CTTGTCGCGCTTGCGCCAG	Xac, Xcc	AAM43336	Endo-1,4-β-xylanase A
xynB	294	TGGGGCAACCAGCAGCCGG	GGTGATGCTGTAGTCGTTGATC	Xac, Xcc, Xoo	AAM43339	Xylanase
II.						
efp	491	TCATCACCGAGACCGAAT	TCCTGGTTGACGAACAG	Xac, Xcc	S. Boudon (*personal communication*)	Elongation factor P
ihfA (syn. gumA)	289	TGACGAAAGCGGAGATGGCCG	TGCCCGGATCCAGCATAAGCC	Xac, Xcc, Xoo	AAA86369	Integration host factor A

[a] DNA fragments correspond to partial genes.

[b] Primer sequences were designed based on multiple sequence alignments of nucleotide or protein sequences (degenerated primers) of genes involved or potentially involved in pathogenicity of the following phytopathogenic bacteria: *Xanthomonas axonopodis* pv. *citri* strain 306 (Xac), *X. campestris* pv. *campestris* strain ATCC 33913 (Xcc), *X. campestris* pv. *vesicatoria* (Xcv) (27), *X. oryzae* pv. *oryzae* strain KACC10331 (Xoo), or *Xylella fastidiosa* strain 9a5c (Xf9a5c) and strain Temecula1 (Xftem).

[c] GenBank accession number (No.) refers to *X. campestris* pv. *campestris* strain ATCC 33913 and *Xylella fastidiosa* strains 9a5c or Temecula1.

[d] Protein names are as described on the National Center for Biotechnology Information (NCBI) Internet site; HLS = hemagglutinin-like secreted, RR = response regulator, and GSP = general secretion pathway.

Total genomic DNA of *X. campestris* pv. *campestris* strain CFBP 5251, *X. campestris* pv. *vesicatoria* strain 91-118, or *Xylella fastidiosa* strain Temecula1 was used systematically as template DNA for a positive or negative control in all PCR amplification series.

Nucleotide sequence analysis of housekeeping genes. Total genomic DNA of nine strains of *Xanthomonas albilineans* differing in pathogenicity in Guadeloupe and *X. albilineans* strain Xa23R1 was used as template DNA to amplify, by PCR, partial housekeeping genes *efp* and *ihfA*. Oligonucleotides used as primers for PCR amplifications are listed in Table 3 and PCR amplifications were performed as described above.

Total genomic DNA of *X. campestris* pv. *campestris* strain CFBP 5251 and *X. arboricola* pv. *pruni* strain CFBP 6653 was used as template DNA for a positive control in all PCR amplifications series.

Phylogenetic analysis. In all, 30 µl of amplicons obtained after PCR amplification of housekeeping genes *efp* and *ihfA* and pathogenicity-related genes *pilB*, *rpfA*, and *xpsE* were sequenced by Genome Express (Meylan, France) using the same primers as used for PCR amplifications. Sequences were aligned and analyzed with DNAMAN Sequence Analysis Software (Lynnon BioSoft, Vaudreuil, Canada). The 5′ and 3′ end sequences of pilB, rpfA1, and xpsE PCR fragments were not obtained for all bacterial strains; therefore, the phylogenetic analyses were performed with the largest sequences common to all strains (644, 859, and 539 bp, respectively). Complete PCR sequences of efp and ihfA PCR fragments were obtained for all bacterial strains and phylogenetic analyses were performed after removal of the primer sequences, leaving 455 and 245 bp, respectively. Jukes-Cantor's model was used to correct distances for multiple substitutions and phylogenetic trees were constructed with the neighbor-joining method (25). Robustness of nodes of the phylogenetic trees was assessed from 1,000 bootstrap resamplings.

Production of diffusible signal molecules. Biosensors *Agrobacterium tumefaciens* NT1 (pZLR4) (11) and *X. campestris* pv. *campestris* B24-B6 (32) and respective control strains were used to detect potential production of different diffusible molecules, *N*-acylhomoserine lactones (AHLs), and PigB diffusible factor (DF) by *X. albilineans* strains. Biosensors were obtained from and were used as described by the respective authors (11,32).

Statistical analysis. Values of DS and ESC each were compared with the General Linear Models procedure of SAS software (version 8.2; SAS Institute Inc., Cary, NC). Cluster analysis based on Euclidean distance was conducted by the UPGMA procedure (45,51). Similarity calculations, cluster analyses, and dendrogram constructions were performed with DARwin program version 4.0 (CIRAD-FLHOR).

RESULTS

Characteristics of the collection of *X. albilineans* strains used in this study. The 75 strains of *X. albilineans* were sampled from 45 sugarcane cultivars and different geographic locations in Guadeloupe. All strains belonged to serovar I as determined by Rott et al. (39), and albicidin production varied among strains of the pathogen (Table 1).

Variation in pathogenicity of *X. albilineans* strains in Guadeloupe. In trial A, 12 weeks after inoculating plants with the 75 strains, DS varied between 0 (46 strains) and 52 (strain GPEPC73) on a scale of 0 to 100. At the end of the experiment, 17 weeks after plant inoculation, ESC varied between 0 (seven strains) and 81.3 (strain GPEPC73), also on a scale of 0 to 100. Nineteen strains representative of this variability were selected arbitrarily for subsequent testing in a second pathogenicity trial (trial B), with more plants inoculated per strain, and statistical analysis of data was performed with these strains in both trials (Table 2).

In trial A, 11 of 19 strains produced symptoms of the disease on noninoculated leaves. No disease symptoms were scored for eight strains at any time on any of the five inoculated plants (data not shown). Twelve weeks after inoculation, DS varied greatly between strains of the pathogen ($P < 0.0001$) and ranged from 0 to 52 (Table 2). Similarly, 17 weeks after plant inoculation, ESC was significantly different between strains ($P < 0.0001$) (Table 2). Four groups of colonization were identified by cluster analysis based on ESC values (Fig. 1): a first group (SCG1) contained nonaggressive or weakly aggressive strains for which ESC varied from 0 (GPEPC17 and GPEPC86) to 6.7 (GPEPC106). A second group (SCG2) consisted of moderately aggressive strains for which ESC ranged from 23.3 (GPEPC91) to 39.7 (GPEPC142). A third group (SCG3) included highly aggressive strains, with ESC values varying from 51 (GPEPC75) to 63.4 (GPEPC143). The fourth group (SCG4) contained the most aggressive strain, with an ESC value of 81.3 (GPEPC73).

In trial B, 14 of 19 strains of the pathogen produced symptoms of the disease on noninoculated leaves. Five strains did not produce disease symptoms at any time on any of the 16 inoculated plants (data not shown). Twelve weeks after inoculation, DS varied significantly between strains of the pathogen ($P < 0.0001$) and ranged from 0 to 78.0 (Table 2).

Sixteen weeks after plant inoculation, stalk colonization varied greatly between strains of the pathogen (Table 2). As in trial A, four groups were identified by cluster analysis based on ESC values (Fig. 1). A first group (SCG1) included weakly aggressive strains with ESC values ranging from 5 (GPEPC101) to 18 (GPEPC86). A second group (SCG2) contained moderately aggressive strains for which ESC ranged from 42 (GPEPC142) to 48 (GPEPC80). A third group (SCG3) included highly aggressive strains, for which ESC values varied from 57 (GPEPC91) to 74 (GPEPC87). A fourth group consisted of the most aggressive strain, with an ESC value of 93 (GPEPC73).

Correlation of DS values ($R = 0.91$) and ESC values ($R = 0.85$) obtained for the 19 strains in trials A and B was high and indicated very good reliability of these results. Additionally, the 19 strains of *X. albilineans* were distributed in four DS groups that were similar to the four stalk colonization groups (data not shown).

Genetic variation in *X. albilineans* from Guadeloupe analyzed by PFGE and Southern hybridization with albicidin biosynthesis genes. Banding patterns were observed for all 20 strains of *X. albilineans* (19 strains from Guadeloupe tested in pathogenicity trials A and B and strain Xa23R1 from Florida) when total genomic DNA was separated by PFGE after enzymatic restriction with *Spe*I. A single banding pattern, corresponding to haplotype B-02 described by Davis et al. (17), was obtained for all strains from Guadeloupe. The banding pattern of strain Xa23R1 was different and corresponded to haplotype A-01, described by Davis et al. (17). Banding patterns also were observed for all strains when total genomic DNA was hybridized with probes pALB571 and pBKS/K7 harboring the albicidin biosynthesis genes, following enzymatic restriction with *Hin*cII. A single pattern was observed for the 19 strains from Guadeloupe which differed from the pattern of strain Xa23R1: 15 DNA bands (ranging from 0.67 to >5.0 kb) and 7 DNA bands (ranging from 0.57 to >6.5 kb) were obtained with probes pALB571 and pBKS/K7, respectively. These banding patterns corresponded to combined haplotype HB2 as described by Champoiseau et al. (12). Banding patterns of Xa23R1 were different and corresponded to combined haplotype HA1.

Genetic variation in *X. albilineans* from Guadeloupe analyzed by AFLP. Nine strains of *X. albilineans* (GPEPC17, GPEPC73, GPEPC75, GPEPC84, GPEPC86, GPEPC87, GPEPC101, GPEPC142, and GPEPC143) from Guadeloupe that were distributed in the same colonization groups in trials A and B, representative of the variability of pathogenicity revealed in this

study, were used to study genetic variation of the pathogen by AFLP (Tables 1 and 2). Banding patterns were observed for all strains and these patterns varied according to the strain of *X. albilineans* following PCR amplification of total genomic DNA with all four selective primer combinations (data not shown). The polymorphic band positions obtained with the four primer combinations were scored and combined for each strain, and six combined haplotypes were defined. These combined haplotypes were distributed into two major genetic groups (AFLP A and AFLP B) by cluster analysis (Fig. 2). In each of these two groups, strains of *X. albilineans* originated from different locations in Guadeloupe and belonged to different groups of stalk colonization (Fig. 1). Similarly, strains belonging to a single haplotype (GA2 or GB3) differed in pathogenicity.

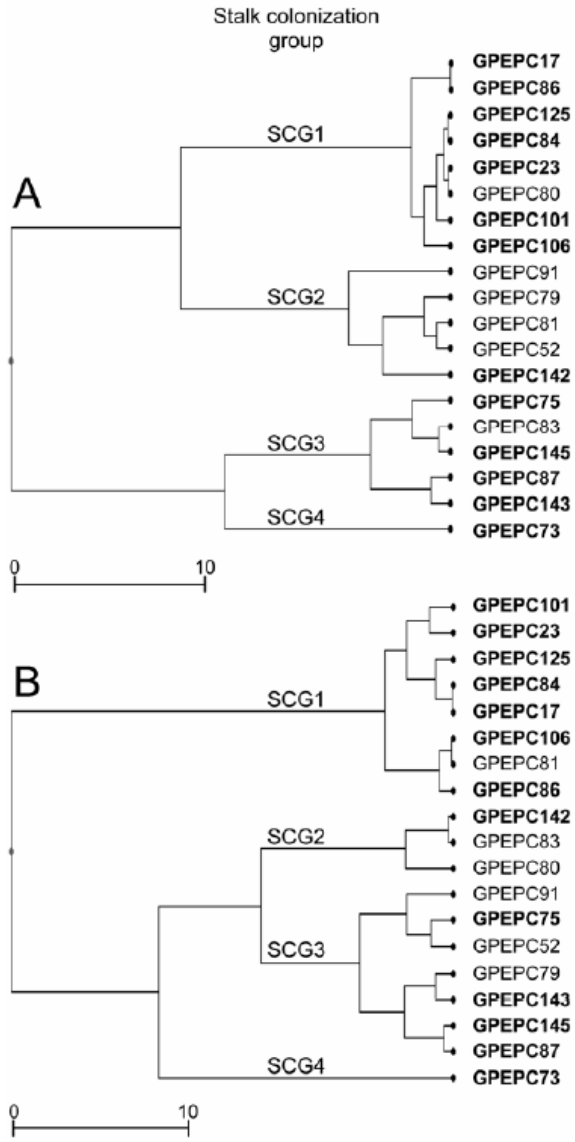

Fig. 1. Unweighted pair group method with arithmetic means dendrograms showing similarity among 19 strains of *Xanthomonas albilineans* based on extent of sugarcane stalk colonization (ESC) 17 and 16 weeks after inoculation of sugarcane cv. B69566 in **A**, trial A and **B**, trial B, respectively. ESC was calculated with bacterial population densities that were determined in every other internode and in all internodes above the location of the three shortened stalk internodes that showed reduced growth after mechanical plant inoculation (cut leaves above the growing point) (SL_0) in **A**, trial A and **B**, trial B, respectively. SCG1 through SCG4 refer to different groups of increasing ESC that were identified in this study. Strain names in bold indicate strains of *X. albilineans* that were distributed in the same groups of stalk colonization in trials A and B. Scale bar represents Euclidean distance between strains based on ESC values.

PCR amplification of pathogenicity-related genes from *X. albilineans*. Single DNA bands of expected sizes were obtained after PCR amplification of total genomic DNA from *X. campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria*, or *X. fastidiosa* strains, used as positive controls, with 40 primer sets (Table 3). Only three DNA fragments (pilB, rpfA1, and xpsE) could be amplified with these primers from total genomic DNA of nine strains of *X. albilineans* from Guadeloupe differing in pathogenicity, and strain Xa23R1 from Florida. No variation was observed in the nucleotide sequence of these DNA fragments among the strains from Guadeloupe; however, eight single synonymous nucleotide mutations (SNM), five SNM and one single nonsynonymous mutation, and four SNM were found between the nine Guadeloupean strains and Floridian strain Xa23R1 for DNA fragments pilB, rpfA1, and xpsE, respectively.

Phylogenetic relationship between *X. albilineans* and other pathogenic and nonpathogenic bacteria. DNA fragments efp and ihfA were amplified from housekeeping genes *efp* and *ihfA* (Table 3). No variation was observed in the nucleotide sequence of these DNA fragments among the nine strains from Guadeloupe and strain Xa23R1 from Florida. All these strains were grouped into a single phylogenetic cluster by phylogenetic analysis with sequences of housekeeping genes *efp* and *ihfA*, and pathogenicity-related genes *pilB*, *rpfA*, and *xpsE* (Fig. 3). Percent identity of the *X. albilineans* strains varied between 85.6 and 90.0% with the cluster containing all the other xanthomonads, and between 74.4 and 79.3% with the two strains of *X. fastidiosa* (Table 4).

Production of diffusible signals. No AHL activity was detected in the supernatant of 5-day-old liquid cultures of the nine strains of *X. albilineans* from Guadeloupe differing in patho-

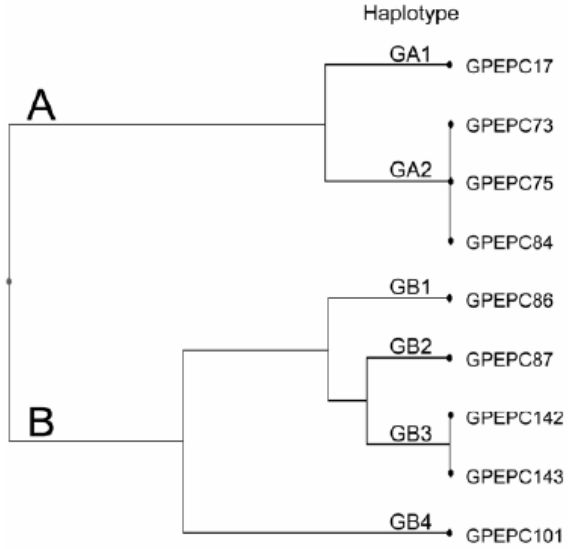

Fig. 2. Genetic relatedness of nine strains of *Xanthomonas albilineans* from Guadeloupe based on amplified fragment length polymorphism (AFLP). After enzymatic restriction with *Sac*I and *Msp*I, total genomic DNA was ligated with specific adapters and amplified by polymerase chain reaction with the selective primer combinations *Sac*I + G/*Msp*I + A, *Sac*I + G/*Msp*I + T, *Sac*I + G/*Msp*I + G, and *Sac*I + G/*Msp*I + CT. Banding patterns obtained with the four primer combinations were combined in six linearly combined haplotypes (GA1, GA2, and GB1 to GB4). Combined haplotypes were used for hierarchical cluster analysis by the unweighted pair group method with arithmetic means, and two AFLP groups (**A** and **B**) were distinguished.

genicity or of strain Xa23R1 from Florida. No DF was detected in cultures of the same 10 *X. albilineans* strains 9 days after inoculation of agar plates with biosensor B24-B6. However, AHLs and DF were detected with the respective *A. tumefaciens*- and *X. campestris* pv. *campestris*-positive controls (data not shown).

Relationship between variation in pathogenicity and genetic or physiological variation of nine strains of *X. albilineans* from Guadeloupe. ESC, DS, genetic variability of the whole genome (by RFLP and AFLP), genetic variability of albicidin biosynthesis genes, and nucleotide sequence of two housekeeping genes and three pathogenicity-related genes were compared for nine strains of *X. albilineans* from Guadeloupe (Table 5). High variation in pathogenicity and four groups of colonization (stalk colonization groups SCG1 to SCG4) were identified. All strains shared the same haplotypes (B-02 and HB2) previously identified by RFLP on the whole genome (17) and with albicidin biosynthesis genes (12), respectively, thus confirming genetic homogeneity of *X. albilineans* strains in Guadeloupe. In contrast, small variation and two genetic groups (AFLP-A [combined haplotypes GA1 and GA2] and AFLP-B [combined haplotypes GB1 to GB4]) were identified by AFLP. However, no relationship between this variation and variation in stalk colonization groups was found. Comparison of nucleotide sequence of two housekeeping genes (*efp* and *ihfA*) and three partial pathogenicity-related genes (*pilB*, *rpfA*, and *xpsE*) revealed no differences among the nine strains from Guadeloupe, but phylogenetic studies confirmed that *X. albilineans* belongs to the genus *Xanthomonas*.

DISCUSSION

In this study, we explored genetic and physiological variability of strains of *X. albilineans* differing in pathogenicity, aiming to identify specific markers associated with different pathotypes of this pathogen. High variation in pathogenicity of 75 strains of *X. albilineans* from Guadeloupe was shown, confirming the existence of different pathotypes of *X. albilineans* in this geographical location (12,15). Surprisingly, most of the 75 strains produced no or only weak disease symptoms after inoculation of sugarcane cv. B69566, which is susceptible to leaf scald. Almost all these strains were sampled from necrotic symptoms on leaves (and different sugarcane cultivars) that were associated with aerial contamination by the pathogen, and that may occur without systemic

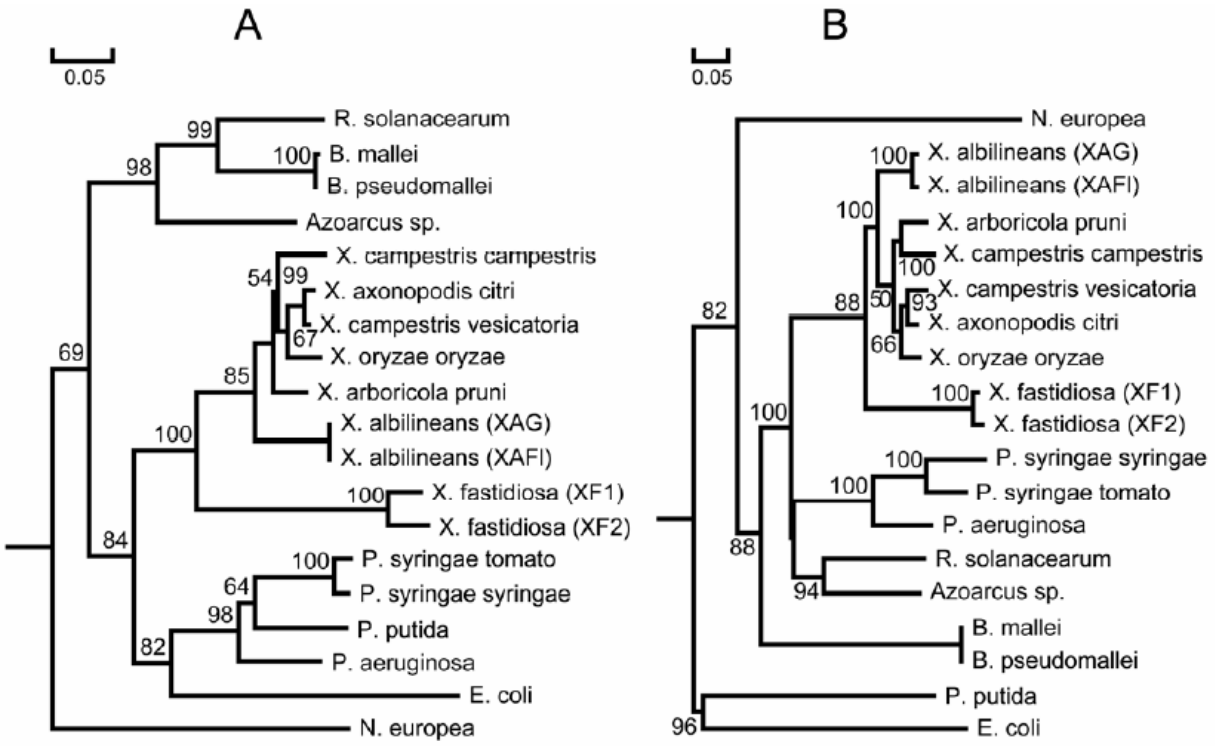

Fig. 3. Neighbor-joining tree of **A**, fragment ihfA (245-bp sequence of partial gene *ihfA*) and **B**, fragment pilB (644-bp sequence corresponding to partial gene *pilB*) nucleotide sequences of 10 strains of *Xanthomonas albilineans* and 17 other bacterial strains (including 1 nonpathogenic and 16 pathogenic strains). DNA fragments from *X. albilineans* strains from Guadeloupe (XAG = GPEPC17, GPEPC73, GPEPC75, GPEPC84, GPEPC86, GPEPC87, GPEPC101, GPEPC142, and GPEPC143), *X. albilineans* strain Xa23R1 (XAFl) from Florida, *X. arboricola* pv. *pruni* strain CFBP 6653, *X. campestris* pv. *campestris* strain ATCC 33913, and *X. campestris* pv. *vesicatoria* strain 91-118 were polymerase chain reaction amplified from total genomic DNA. Nucleotide sequence from other strains are from GenBank (accession no.; position): *Azoarcus* strain EbN1 (A, CR555306, 504922-504611; B, CR555306, 2574125-2572410), *Burkholderia mallei* strain ATCC 23344 (A, CP000010, 1133153-1133557; B, CP000010, 2635324-2636583), *B. pseudomallei* strain K96243 (A, BX571965, 2309571-2309224; B, BX571965, 3584982-3586241), *Escherichia coli* strain K-12 MG1655 (A, U00096, 1793576-1793277; B, U00096, 117099-115714), *Nitrosomas europea* ATCC 19718 (A, BX321859, 116386-116078; B, BX321861, 195575-193884), *Pseudomonas aeruginosa* strain PAO1 (A, AE004702, 1576-1274; B, AE004866, 9737-11437), *P. putida* strain KT2440 (A, AE016783, 101086-101388; B, AE016793, 185393-187066), *P. syringae* pv. *syringae* strain B728a (A, CP000075, 2522380-2522682; B, CP000075, 905203-903509), *P. syringae* pv. *tomato* strain DC3000 (A, AE016853, 2634012-2634314; B, AE016853, 1006981-1005287), *Ralstonia solanacearum* strain GMI1000 (A, AL646065, 87590-87904; B, AL646072, 26520-28247), *Xanthomonas axonopodis* pv. *citri* strain 306 (A, AE011899, 1068-769; B, AE011969, 10306-8570), *Xyllela fastidiosa* strain 9a5c (XF1) (A, AE003916, 5155-5454; B, AE004061, 3126-4859), *X. fastidiosa* strain Temecula1 (XF2) (A, AE012560, 134145-133856; B, AE012560, 153609-155342), and *Xanthomonas oryzae* pv. *oryzae* strain KACC10331 (A, AE013598, 3417819-3417520; B, AE013598, 1628312-1630099). Bootstrap values for 1,000 resamplings are shown as percentage. Similar phylogenetic trees were obtained with the nucleotide sequences from *efp*, *rpfA*, and *xpsE* partial genes (data not shown). Scale bar units are in number of substitutions per nucleotide.

colonization of the sugarcane stalk (13,15). These data suggested that different mechanisms are involved in the capacity of the pathogen to colonize the leaf surface, with subsequent penetration into the host, and in the capacity to systemically colonize the sugarcane stalk, with subsequent systemic disease symptoms.

DS and ESC were characterized further for 19 of the 75 strains of the pathogen. Of the 19 strains, 13 were distributed among the same stalk colonization groups (groups SCG1 to SCG4) in two independent greenhouse experiments, indicating reliability of results (Fig. 1). Strains from a single geographic (field) location or isolated from the same sugarcane cultivar were distributed in different stalk colonization or DS groups. In contrast, strains from different geographic locations or sugarcane cultivars were distributed in the same stalk colonization or DS groups. Therefore, large differences in pathogenicity of *X. albilineans* can exist among strains of the pathogen, not only between different countries or geographical areas (12) but also within a single sugarcane field.

No genetic variation was identified among 19 pathogenic variants of *X. albilineans*, isolated in 2003 in Guadeloupe, by RFLP based on the whole genome or on albicidin biosynthesis genes. This result confirmed genetic homogeneity of recent populations of *X. albilineans* in Guadeloupe, although several genetic variants, serovars, and pathotypes have been reported in the past on this island (12,15,17). Similar variation in pathogenicity or aggressiveness of genetically related strains was found by different DNA-based methods in several other plant-pathogenic bacteria (24,29,30). In contrast, different strains of *Ralstonia solanacearum* were differentiated by AFLP, and especially strains that were not distinguished by an RFLP-based technique (33). Additionally, AFLP was used successfully to identify bacterial pathogenicity islands, genes involved in pathogenic fitness, and regulators of virulence of the bacterial pathogen *X. axonopodis* pv. *manihotis* after excision, amplification, cloning, and sequence homology analysis of polymorphic bands from AFLP gels (22). In this study, AFLP primer combinations *Sac*I + G/*Msp*I + A, *Sac*I + G/*Msp*I + T, *Sac*I + G/*Msp*I + G, and *Sac*I + G/*Msp*I + CT also were efficient in identifying genetic variability within *X. albilineans* in Guadeloupe. Differences in gel resolution and number of polymorphic bands were observed between the two independent replications of the AFLP assay. However, the number of haplotypes and strain assignment to these haplotypes did not vary, indicating reliability of the technique (data not shown). Six combined haplotypes and two major genetic groups, AFLP-A (combined haplotypes GA1 and GA2) and AFLP-B (combined haplotypes GB1 to GB4), were obtained, but no relationship with the stalk colonization groups or DS groups was found (Table 5).

Single nucleotide variations in the sequence of pathogenicity-related genes has been shown to be responsible for variation in virulence or aggressiveness in several pathogenic microorganisms (3,47,52). In bacterial plant pathogens, a wide range of pathogenicity-related genes have been identified and analyzed. These genes code for secretion system constitutive proteins, exopolysaccharides, virulence factors, toxins, plant cell-wall-degrading enzymes, cell mobility and motility factors, or adhesion factors (21,48). PCR amplification with specific or degenerate primers has been used successfully to identify homologous gene sequences at pathovar or even generic levels in *Pseudomonas* and *Xanthomonas* spp. (23,27,43). However, of 40 pathogenicity-related genes from several xanthomonads or *Xylella fastidiosa*, only 3 (*pilB*, *rpfA*, and *xpsE*) were amplified in *Xanthomonas albilineans*. These genes all are involved in general pathogenicity mechanisms. Gene *pilB* codes for the type IV pilus biogenesis protein B involved in twitching motility, *rpfA* encodes an aconitase involved in the regulator system of pathogenicity factor, and *xpsE* codes for the type II secretion system biogenesis protein E involved in a general secretory pathway in several plant pathogens (10,14,19,44). Additionally, among nine strains of *X. albilineans* differing in pathogenicity, no variation was found in the nucleotide sequence of these fragments. This result confirmed, at the nucleotide sequence level, the genetic uniformity of this pathogen in Guadeloupe. Absence of amplification with 37 primer combinations suggested that the corresponding genes involved in pathogenicity either are absent in *X. albilineans* or differ from those existing in other closely related pathogens. No amplification in *X. albilineans* of *hrcL*, *hrcN*, and *hrpB7* genes coding for essential components of the common type III secretion system in plant-pathogenic bacteria confirmed previous results (27).

Housekeeping genes are highly conserved genes and, therefore, are very useful for phylogenetic studies of a pathogen (9). The nucleotide sequence of housekeeping genes *efp* and *ihfA* did not vary among nine strains in Guadeloupe differing in pathogenicity. However, the phylogenetic study of 26 bacterial species (including *X. albilineans*) with these two housekeeping genes and

TABLE 4. Nucleotide sequence identity (%) within and among groups of strains of six xanthomonads and *Xylella fastidiosa*

Groups of strains[a]	*X. albilineans*	Xantho	*Xylella*
I. Fragment efp			
X. albilineans	100	…	…
Xantho	86.1–87.6	94.1–98.2	…
Xylella	76.2–77.0	76.6–79.4	98.2
II. Fragment ihfA			
X. albilineans	100	…	…
Xantho	88.6–90.0	92.7–98.6	…
Xylella	74.4–76.1	73.4–78.5	94.1
III. Fragment pilB			
X. albilineans	98.8–100	…	…
Xantho	87.9–89.6	91.6–96.0	…
Xylella	78.6–79.3	78.7–80.5	97.7
IV. Fragment rpfA1[b]			
X. albilineans	99.4–100	…	…
Xantho	85.6–86.6	91.5–97.2	…
Xylella	78.7–79.3	78.5–79.8	98.6
V. Fragment xpsE[b]			
X. albilineans	99.3–100	…	…
Xantho	88.3–89.6	93.7–97.2	…
Xylella	75.3–75.7	74.8–76.6	97.0

[a] *X. albilineans* = nine strains of *Xanthomonas albilineans* from Guadeloupe and strain Xa23R1 from Florida; Xantho = strains *X. arboricola* pv. *pruni*, *X. axonopodis* pv. *citri*, *X. campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria*, and *X. oryzae* pv. *oryzae*; and *Xylella* = *Xylella fastidiosa* strain 9a5c and *X. fastidiosa* strain Temecula1.

[b] Sequence of this fragment was not available for *Xanthomonas arboricola* pv. *pruni*.

TABLE 5. Summary of genetic and pathogenic characteristics of nine strains of *Xanthomonas albilineans* from Guadeloupe

			Combined haplotype	
Strain[a]	Stalk group[b]	RFLP-PFGE[c]	ALB-RFLP[d]	AFLP[e]
GPEPC17	SCG1	B-02	HB2	GA1
GPEPC73	SCG4	B-02	HB2	GA2
GPEPC75	SCG3	B-02	HB2	GA2
GPEPC84	SCG1	B-02	HB2	GA2
GPEPC86	SCG1	B-02	HB2	GB1
GPEPC87	SCG3	B-02	HB2	GB2
GPEPC101	SCG1	B-02	HB2	GB4
GPEPC142	SCG2	B-02	HB2	GB3
GPEPC143	SCG3	B-02	HB2	GB3

[a] Origin and characteristics of strains are shown in Table 1. All strains showed an identical nucleotide sequence for housekeeping genes *efp* and *ihfA*, and pathogenicity related genes *pilB*, *rpfA*, and *xpsE*.

[b] Stalk colonization group; data from Figure 1. Disease severity groups were similar to stalk colonization groups.

[c] Haplotype restriction fragment length polymorphism (RFLP) pulsed-field gel electrophoresis (PFGE); from this study and Davis et al. (17).

[d] Albicidin (ALB)-RFLP; from this study and Champoiseau et al. (12).

[e] Amplified fragment length polymorphism (AFLP); this study.

pathogenicity genes *pilB*, *rpfA*, and *xpsE* confirmed that *X. albilineans* was closest to the *X. campestris* group (34), but showed also that *X. albilineans* was related to *X. fastidiosa*, another xylem pathogen that does not possess any *avr* or *hrp* genes (44). Very little information is available presently regarding genes involved in pathogenicity of *Xylella fastidiosa*; however, based on their phylogenetic positions, it can be hypothesized that *Xanthomonas albilineans* and this pathogen share common genetic features, including those involved in pathogenicity.

Autoinduction, or quorum sensing, is a feature of virulence of plant-pathogenic bacteria that involves secretion and detection of small, diffusible signal molecules that permit cell-to-cell communication and allows bacteria to assess their local population density or physical confinement (49). Traits regulated by quorum sensing include the production of extracellular polysaccharides, degradative enzymes, antibiotics, siderophores, and pigments, as well as Hrp protein secretion, Ti plasmid transfer, motility, biofilm formation, and epiphytic fitness (49). AHLs are the most commonly reported type of quorum-sensing signals. No evidence of AHL production was detected in nine strains of *X. albilineans* from Guadeloupe differing in pathogenicity. Similarly, no AHL production was found in two and seven different pathovars of *X. oryzae* and *X. campestris*, respectively (11). However, it cannot be excluded that other quorum-sensing or cell-to-cell communication-diffusible signal molecules play a role in the regulation of several pathogenicity traits in *X. albilineans*. These molecules may explain, at least in part, variation in pathogenicity of *X. albilineans*, as was shown recently for *X. campestris* pv. *campestris* (50).

In conclusion, the origin of variation in pathogenicity of genetically closely related strains of *X. albilineans* remains to be deciphered. Genetic uniformity of strains of this pathogen differing in pathogenicity was observed at different genetic levels, from the whole genome to specific nucleotide sequences. However, only a small part of the genome has been investigated in details. Because no large deletion or insertion was detected between the genomes of *X. albilineans* pathotypes, apart from complex non-genetic regulatory systems not identified so far in this pathogen, single nucleotide mutations in one or more genes involved in pathogenicity of *X. albilineans* most likely are responsible for variation in pathogenicity of *X. albilineans*. Therefore, analysis of the complete genome sequence of *X. albilineans* and comparison with genome sequences of other phytopathogenic bacteria might be the next step in the identification of pathogenicity genes in this pathogen. Sequencing of the genome of *X. albilineans* strain GPEPC73, isolated in this study, is in progress at Genoscope (Evry, France). Obtaining the entire genome sequence of this pathogen will lead to a significant breakthrough in the characterization of the molecular mechanisms involved in the sugarcane–*X. albilineans* interaction.

ACKNOWLEDGMENTS

This work was supported in part by the "Conseil Régional de Guadeloupe" and the "Fonds Social Européen". We thank R. Boisne-Noc, S. Duplan, S. Joseph, L. Toubi, and E. Vivien for technical assistance and advice; S. Boudon for supplying efp oligonucleotides; S. K. Farrand for supplying AHL biosensor strains; D. W. Gabriel for supplying *Xylella fastidiosa* DNA; A. R. Poplawsky for supplying PigB DF biosensor strains; and M. J. Davis for critical review of the manuscript.

LITERATURE CITED

1. Astua-Monge, G., Minsavage, G. V., Stall, R. E., Davis, M. J., Bonas, U., and Jones, J. B. 2000. Resistance of tomato and pepper to T3 strains of *Xanthomonas campestris* pv. *vesicatoria* is specified by a plant-inducible avirulence gene. Mol. Plant-Microbe Interact. 13:911-921.
2. Autrey, L. J. C., Saumtally, S., Dookun, A., Sullivan, S., and Dhayan, S. 1995. Aerial transmission of the leaf scald pathogen, *Xanthomonas albilineans*. Proc. Int. Soc. Sugar Cane Technol. 21:508-526.
3. Bender, C. L., Alarcon-Chaidez, F., and Gross, D. C. 1999. *Pseudomonas syringae* phytotoxins: Mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol. Mol. Biol. Rev. 63:266-292.
4. Birch, R. G. 2001. *Xanthomonas albilineans* and the antipathogenesis approach to disease control. Mol. Plant Pathol. 2:1-11.
5. Birch, R. G., and Patil, S. S. June 1985. Antibiotic and process for the production thereof. U. S. patent 4,525,354.
6. Birch, R. G., and Patil, S. S. 1985. Preliminary characterization of an antibiotic produced by *Xanthomonas albilineans* which inhibits DNA synthesis in *Escherichia coli*. J. Gen. Microbiol. 131:1069-1075.
7. Birch, R. G., and Patil, S. S. 1987. Correlation between albicidin production and chlorosis induction by *Xanthomonas albilineans*, the sugarcane pathogen. Physiol. Mol. Plant Pathol. 30:199-206.
8. Birch, R. G., and Patil, S. S. 1987. Evidence that an albicidin-like phytotoxin induces chlorosis in sugarcane leaf scald disease by blocking plastid DNA replication. Physiol. Mol. Plant Pathol. 30:207-214.
9. Boudon, S., Manceau, C., and Nottéghem, J.-L. 2005. Structure and origin of *Xanthomonas arboricola* pv. *pruni* populations causing bacterial spot of stone fruit trees in Western Europe. Phytopathology 95:1081-1088.
10. Cao, H., Baldini, R. L., and Rahme, L. G. 2001. Common mechanisms for pathogens of plants and animals. Annu. Rev. Phytopathol. 39:259-284.
11. Cha, C., Gao, P., Chen, Y.-C., Shaw, P. D., and Farrand, S. K. 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol. Plant-Microbe Interact. 11:1119-1129.
12. Champoiseau, P., Daugrois, J.-H., Girard, J.-C., Royer, M., and Rott, P. 2006. Variation in albicidin biosynthesis genes and in pathogenicity of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. Phytopathology 96:33-45.
13. Comstock, J. C. 2001. Foliar symptoms of sugarcane leaf scald. Sugar J. 64:23-32.
14. Da Silva, A. C. R., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Van Sluys, M. A., Almeida, N. F., Alves, L. M. C., do Amaral, A. M., Bertolini, M. C., Camargo, L. E. A., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L. P., Cicarelli, R. M. B., Coutinho, L. L., Cursino-Santos, J. R., El-Dorry, H., Faria, J. B., Ferreira, A. J. S., Ferreira, R. C. C., Ferro, M. I. T., Formighieri, E. F., Franco, M. C., Greggio, C. C., Gruber, A., Katsuyama, A. M., Kishi, L. T., Leite, R. P., Lemos, E. G. M., Lemos, M. V. F., Locali, E. C., Machado, M. A., Madeira, A. M. B. N., Martinez-Rossi, N. M., Martins, E. C., Meidanis, J., Menck, C. F. M., Miyaki, C. Y., Moon, D. H., Moreira, L. M., Novo, M. T. M., Okura, V. K., Oliveira, M. C., Oliveira, V. R., Pereira, H. A., Rossi, A., Sena, J. A. D., Silva, C., de Souza, R. F., Spinola, L. A. F., Takita, M. A., Tamura, R. E., Teixeira, E. C., Tezza, R. I. D., Trindade dos Santos, M., Truffi, D., Tsai, S. M., White, F. F., Setubal, J. C., and Kitajima, J. P. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature 417:459-463.
15. Daugrois, J. H., Dumont, V., Champoiseau, P., Costet, L., Boisne-Noc, R., and Rott, P. 2003. Aerial contamination of sugarcane in Guadeloupe by two strains of *Xanthomonas albilineans*. Eur. J. Plant Pathol. 109: 445-458.
16. Davis, M. J., Rott, P., Baudin, P., and Dean, J. L. 1994. Evaluation of selective media and immunoassays for detection of *Xanthomonas albilineans*, causal agent of sugarcane leaf scald disease. Plant Dis. 78:78-82.
17. Davis, M. J., Rott, P., Warmuth, C. J., Chatenet, M., and Baudin, P. 1997. Intraspecific genomic variation within *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. Phytopathology 87:316-324.
18. Diaz, M., Peralta, E. L., Iglesias, A., Pazos, V., Carvajal, O., Perez, J., Gigliotti, E. A., Gagliardi, P. R., Wendland, A., and Camargo, L. E. A. 2001. *Xanthomonas albilineans* haplotype B responsible for a recent sugarcane leaf scald disease outbreak in Cuba. Plant Dis. 85:334.
19. Dow, J. M., Feng, J.-X., Barber, C. E., Tang, J.-L., and Daniels, M. J. 2000. Novel genes involved in the regulation of pathogenicity factor production within the *rpf* gene cluster of *Xanthomonas campestris*. Microbiology 146:885-891.
20. Feldmann, P., Sapotille, J., Grédoire, P., and Rott, P. 1994. Micropropagation of sugarcane. Pages 15-17 in: In vitro Culture of Tropical Plants. C. Teisson, ed. La Librairie du Cirad, Montpellier, France.
21. Genin, S., and Boucher, C. 2004. Lessons learned from the genome analysis of *Ralstonia solanacearum*. Annu. Rev. Phytopathol. 42:107-134.
22. Gonzalez, C., Restrepo, S., Tohme, J., and Verdier, V. 2002. Characterization of pathogenic and nonpathogenic strains of *Xanthomonas axonopodis* pv. *manihotis* by PCR-based DNA fingerprinting techniques. FEMS Microbiol. Lett. 215:23-31.
23. Gropp, S. J., and Guttman, D. S. 2004. The PCR amplification and characterization of entire *Pseudomonas syringae* hrp/hrc clusters. Mol. Plant Pathol. 5:137-140.

24. Horita, M., and Tsuchiya, K. 2000. Genetic diversity of Japanese strains of *Ralstonia solanacearum*. Phytopathology 91:399-407.
25. Jukes, T. H., and Cantor, C. R. 1969. Evolution of protein molecules. Pages 21-132 in: Mammalian Protein Metabolism. H. N. Munro, ed. Academic Press, New York.
26. Koike, H. 1965. The aluminum cap method for testing sugarcane varieties against leaf scald disease. Phytopathology 55:317-319.
27. Leite, R. P. J., Minsavage, G. V., Bonas, U., and Stall, R. E. 1994. Detection and identification of phytopathogenic *Xanthomonas* strains by identification of DNA sequences related to the *hrp* genes of *Xanthomonas campestris* pv. *vesicatoria*. Appl. Environ. Microbiol. 60:1068-1077.
28. Mohamed, I. S., Rott, P., Davis, M. J., and Chatenet, M. 1996. Differentiation of *Xanthomonas albilineans* strains based on multiplication of the pathogen in sugarcane varieties. Proc. Int. Soc. Sugar Cane Technol. 22:486-492.
29. Mundt, C. C., Nieva, L. P., and Vera Cruz, C. M. 2002. Variation for aggressiveness within and between lineages of *Xanthomonas oryzae* pv. *oryzae*. Plant Pathol. 51:163-168.
30. Nelson, R. J., Baraoidan, M. R., Vera Cruz, C. M., Yap, I. V., Leach, J. E., Mew, T. W., and Leung, H. 1994. Relationship between phylogeny and pathotype for the bacterial pathogen of rice. Appl. Environ. Microbiol. 60:3275-3283.
31. Pilet, F., Clerc, F., Royer, M., Chatenet, M., Trigalet-Demery, D., and Rott, P. 2000. Différenciation des *Xanthomonas* pathogènes de la canne à sucre par RFLP et AFLP. Page 48 in: Résumés des Quatrièmes Rencontres de Phytobactériologie. CNRS/INRA, Aussois, France.
32. Poplawsky, A. R., and Chun, W. 1997. *pigB* determines a diffusible factor needed for extracellular polysaccharide slime and Xanthomonadin production in *Xanthomonas campestris* pv. *campestris*. J. Bacteriol. 179:439-444.
33. Poussier, S., Trigalet-Demery, D., Vandewalle, P., Goffinet, B., Luisetti, J., and Trigalet, A. 2000. Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the *hrp* gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. Microbiology 146:1679-1692.
34. Rademaker, J. L. W., Louws, F. J., Schultz, M. H., Rossbach, U., Vauterin, L., Swings, J., and de Bruijn, F. J. 2005. A comprehensive species to strain taxonomic framework for *Xanthomonas*. Phytopathology 95:1098-1111.
35. Rott, P., Chatenet, M., Granier, M., and Baudin, P. 1988. L'échaudure des feuilles de la canne à sucre provoquée par *Xanthomonas albilineans* (Ashby) Dowson. II. Diagnostic et spectres d'hôtes de l'agent pathogène en Afrique tropicale. Agron. Trop. (Paris) 43:244-252.
36. Rott, P., Costet, L., Davis, M. J., Frutos, R., and Dean, G. 1996. At least two separate gene clusters are involved in albicidin production by *Xanthomonas albilineans*. J. Bacteriol. 178:4590-4596.
37. Rott, P., and Davis, M. J. 1996. Recent advances in research on variability of *Xanthomonas albilineans*, causal agent of sugarcane leaf scald disease. Proc. Int. Soc. Sugar Cane Technol. 22:498-504.
38. Rott, P., and Davis, M. J. 2000. Leaf scald. Pages 38-44 in: A Guide to Sugarcane Diseases. P. Rott, R. A. Bailey, J. C. Comstock, B. J. Croft, and A. S. Saumtally, eds. La librairie du Cirad, Montpellier, France.
39. Rott, P., Davis, M. J., and Baudin, P. 1994. Serological variability in *Xanthomonas albilineans*, causal agent of leaf scald disease of sugarcane. Plant Pathol. 43:344-349.
40. Rott, P., Mohamed, I. S., Klett, P., Soupa, D., de Saint-Albin, A., Feldmann, P., and Letourmy, P. 1997. Resistance to leaf scald disease is associated with limited colonization of sugarcane and wild relatives by *Xanthomonas albilineans*. Phytopathology 87:1202-1213.
41. Royer, M., Costet, L., Vivien, E., Bes, M., Cousin, A., Damais, A., Pieretti, I., Savin, A., Megessier, S., Viard, M., Frutos, R., Gabriel, D. W., and Rott, P. 2004. Albicidin pathotoxin produced by *Xanthomonas albilineans* is encoded by three large PKS and NRPS genes present in a gene cluster also containing several putative modifying, regulatory, and resistance genes. Mol. Plant-Microbe Interact. 17:414-427.
42. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
43. Seal, S. E., Jackson, L. A., and Daniels, M. J. 1992. Isolation of a *Pseudomonas solanacearum*-specific DNA probe by subtraction hybridization and construction of species-specific oligonucleotide primers for sensitive detection by the polymerase chain reaction. Appl. Environ. Microbiol. 58:3751-3758.
44. Simpson, A. J. G., Reinach, F. C., Arruda, P., Abreu, F. A., Acencio, M., Alvarenga, R., Alves, L. M. C., Araya, J. E., Baia, G. S., Baptista, C. S., Barros, M. H., Bonaccorsi, E. D., Bordin, S., Bové, J. M., Briones, M. R. S., Bueno, M. R. P., Camargo, A. A., Camargo, L. E. A., Carraro, D. M., Carrer, H., Colauto, N. B., Colombo, C., Costa, F. F., Costa, M. C. R., Costa-Neto, C. M., Coutinho, L. L., Cristofani, M., Dias-Neto, E., Docena, C., El-Dorry, H., Facincani, A. P., Ferreira, A. J. S., Ferreira, V. C. A., Ferro, J. A., Fraga, J. S., França, S. C., Franco, M. C., Frohme, M., Furlan, L. R., Garnier, M., Goldman, G. H., Goldman, M. H. S., Gomes, S. L., Gruber, A., Ho, P. L., Hoheisel, J. D., Junqueira, M. L., Kemper, E. L., Kitajima, J. P., Krieger, J. E., Kuramae, E. E., Laigret, F., Lambais, M. R., Leite, L. C. C., Lemos, E. G. M., Lemos, M. V. F., Lopes, S. A., Lopes, C. R., Machado, J. A., Machado, M. A., Madeira, A. M. B. N., Madeira, H. M. F., Marino, C. L., Marques, M. V., Martins, E. A. L., Martins, E. M. F., Matsukuma, A. Y., Menck, C. F. M., Miracca, E. C., Miyaki, C. Y., Monteiro-Vitorello, C. B., Moon, D. H., Nagai, M. A., Nascimento, A. L. T. O., Netto, L. E. S., Nhani, A., Nobrega, F. G., Nunes, L. R., Oliveira, M. A., de Oliveira, M. C., de Oliveira, R. C., Palmieri, D. A., Paris, A., Peixoto, B. R., Pereira, G. A. G., Pereira, H. A., Pesquero, J. B., Quaggio, R. B., Roberto, P. G., Rodrigues, V., de M. Rosa, A. J., de Rosa, V. E., de Sá, R. G., Santelli, R. V., Sawasaki, H. E., da Silva, A. C. R., da Silva, A. M., da Silva, F. R., Silva, W. A., da Silveira, J. F., Silvestri, M. L. Z., Siqueira, W. J., de Souza, A. A., de Souza, A. P., Terenzi, M. F., Truffi, D., Tsai, S. M., Tsuhako, M. H., Vallada, H., Van Sluys, M. A., Verjovski-Almeida, S., Vettore, A. L., Zago, M. A., Zatz, M., Meidanis, J., and Setubal, J. C. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406:151-157.
45. Sneath, P. H. A., and Sokal, R. R. 1973. Numerical Taxonomy: The Principles and Practice of Numerical Classification, W. H. Freeman and Co., San Francisco.
46. Titarenko, E., Lopez-Solanilla, E., Garcia-Olmedo, F., and Rodriguez-Palenzuela, P. 1997. Mutants of *Ralstonia* (*Pseudomonas*) *solanacearum* sensitive to antimicrobial peptides are altered in their lipopolysaccharide structure and are avirulent in tobacco. J. Bacteriol. 179:6699-6704.
47. Van den Broek, P., Pittet, A., and Hajjaj, H. 2001. Aflatoxin genes and the aflatoxigenic potential of Koji moulds. Appl. Microbiol. Biotechnol. 57:192-199.
48. Van Sluys, M. A., Monteiro-Vitorello, C. B., Camargo, L. E. A., Menck, C. F. M., da Silva, A. C. R., Ferro, J. A., Oliveira, M. C., Setubal, J. C., Kitajima, J. P., and Simpson, A. J. 2002. Comparative genomic analysis of plant-associated bacteria. Annu. Rev. Phytopathol. 40:169-189.
49. Von Bodman, S. B., Bauer, W. D., and Coplin, D. L. 2003. Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol. 41:455-482.
50. Wang, L.-H., He, Y., Gao, Y., Wu, J. E., Dong, Y.-H., He, C., Wang, S. X., Weng, L.-X., Xu, J.-L., Tay, L., Fang, R. X., and Zhang, L.-H. 2004. A bacterial cell-cell communication signal with cross-kingdom structural analogues. Mol. Microbiol. 51:903-912.
51. Ward, J. H. 1963. Hierarchical grouping to optimize an objective function. Am. Stat. Assoc. J. 58:236-244.
52. Weiland, J. J., and Edwards, M. C. 1996. A single nucleotide substitution in the alpha-a gene confers oat pathogenicity to barley stripe mosaic virus strain ND18. Mol. Plant-Microbe Interact. 9:62-67.
53. Zhang, L., Xu, J., and Birch, R. G. 1999. Engineered detoxification confers resistance against a pathogenic bacterium. Nat. Biotechnol. 17:1021-1024.

Variation in Infection Capacity and in Virulence Exists Between Genotypes of *Sugarcane yellow leaf virus*

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ABSTRACT

Abu Ahmad, Y., Costet, L., Daugrois, J.-H., Nibouche, S., Letourmy, P., Girard, J.-C., and Rott, P. 2007. Variation in infection capacity and in virulence exists between genotypes of *Sugarcane yellow leaf virus*. Plant Dis. 91:253-259.

Two experiments, one in Guadeloupe and one in Réunion Island, were performed to transmit different genotypes of *Sugarcane yellow leaf virus* (SCYLV) to eight sugarcane cultivars differing in resistance to infection by the virus and to yellow leaf. Transmission was attempted from SCYLV-infected sugarcane plants or leaves to healthy tissue-cultured plantlets grown in vitro and with the aphid vector *Melanaphis sacchari*. After inoculation and elimination of insects with an insecticide, plantlets were transferred to Montpellier, France and grown in a greenhouse. Plants were tested for presence of SCYLV by tissue-blot immunoassay and reverse-transcription polymerase chain reaction after 5 to 6 months of growth. SCYLV genotypes BRA-PER, CUB, and REU were detected in 47, 62, and 39% of plants inoculated with these genotypes in Guadeloupe, respectively. SCYLV genotypes BRA-PER and REU and a mixed infection of genotypes BRA-PER and REU were detected in 56, 33, and 42% of plants inoculated with these genotypes in Réunion Island, respectively. Genotypes BRA-PER and CUB could be transmitted to all eight sugarcane cultivars, but genotype REU could never be transmitted to resistant sugarcane cvs. H78-4153 and H78-3567. SCYLV genotype REU was transmitted successfully to sugarcane cv. R570 in Guadeloupe, but not in Réunion Island. Genotypes BRA-PER and CUB induced yellow leaf symptoms in susceptible or highly susceptible sugarcane cultivars, whereas genotype REU induced very few symptoms. SCYLV was not found in several symptomatic plants, suggesting an association of disease with undetectable populations of the virus or a nonviral cause. This is the first report of variation in infection capacity and in virulence of SCYLV.

Additional keywords: *Luteoviridae*, *Polerovirus*

Yellow leaf of sugarcane (*Saccharum* spp. hybrids) is caused by *Sugarcane yellow leaf virus* (SCYLV) and diseased plants may exhibit intense yellowing of the leaf midrib. This discoloration of leaves often occurs while the lamina is still green. Symptoms also include shortening of terminal internodes, yellowing, and necrosis of older leaves. However, most of these symptoms may be related to other biotic or abiotic factors (17,19). The disease is also associated with reduction in sucrose content in stalks, accumulation of sucrose in leaf midribs, and yield losses (10,11,15, 16,28,33). Incidence of SCYLV in commercial fields can reach 100% in susceptible cultivars (7,27,35), and hot-water treatment of cuttings is not effective in eliminating the virus from infected material (6,25). Because infected plants are often asymptomatic, SCYLV was spread worldwide through infected germplasm before diagnostic methods were developed (6,18,29,31). Resistance to SCYLV has been shown to occur in sugarcane cultivars and it appears to be the most promising means to control the disease (31,34).

Sequence homology studies suggested that SCYLV arose by interspecies recombination between members of the genera *Luteovirus* and *Polerovirus* (24,32). Therefore, SCYLV recently has been assigned to the genus *Polerovirus* of the family *Luteoviridae* (8). Members of *Luteoviridae* have ssRNA genomes, reside in the phloem tissue of plants, and are not transmitted mechanically (13,22). SCYLV was transmitted successfully to healthy sugarcane plants by two aphid species that colonize sugarcane: *Melanaphis sacchari* and *Rhopalosiphum maidis* (*Homoptera: Aphididae*) (29,31). Transmission of members of *Luteoviridae* by these insects generally occurs in a persistent, circulative, and nonreplicative manner (12,13).

Several genetic diversity studies showed that SCYLV was a variable virus (1,5,23). Abu Ahmad and co-workers (1,2) described the occurrence and geographical distribution of four SCYLV genotype groups called genotypes BRA, CUB, PER, and REU. BRA-PER, a combined genotype that represents genotypes BRA and genotype PER that are relatively close phylogenetically (1) and that could not be differentiated by specific reverse-transcription polymerase chain reaction (RT-PCR) primers (2), is the most widespread. It was found in 18 different geographical locations all around the world. In contrast, genotypes CUB and REU were found in only 4 of 18 geographical locations. In most locations, plants were infected by only one of these genotypes, but in others, two or more genotypes were detected. Genotypes BRA-PER, CUB, and REU all were found in Guadeloupe in locally bred cultivars, suggesting local transmission of all three genotypes. Genotypes BRA-PER and REU were found in Réunion Island, but REU was the only one that was detected in locally bred and commercially grown cultivars.

The objective of this study was to investigate the biological significance of the occurrence of different genotypes of SCYLV, with special emphasis on infection capacity (based on transmission and infection rate) and virulence (capacity to cause leaf symptoms) of the virus. SCYLV genotypes BRA-PER, CUB, and REU in Guadeloupe and genotypes BRA-PER and REU in Réunion Island were transmitted locally via *M. sacchari* to in vitro tissue-cultured plants of eight sugarcane cultivars differing in resistance to infection by SCYLV and resistance to yellow leaf. Inoculated plants then were transferred to Montpellier, France and grown in a greenhouse to analyze efficiency of transmission in a vector-free environment. Analysis of

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Accepted for publication 19 September 2006.

DOI: 10.1094/PDIS-91-3-0253

incidence of the different SCYLV genotypes in the eight sugarcane cultivars showed the existence of different strains within this virus species.

MATERIALS AND METHODS

Production of SCYLV-free tissue-cultured sugarcane plantlets. Cuttings of eight sugarcane cultivars differing in resistance to infection by SCYLV or to yellow leaf were imported to Montpellier (Table 1). These cultivars were grown in pots in a greenhouse and meristem cultures were prepared as previously described (6). Tissue-cultured plantlets were grown and propagated at 28ºC in 200-by-25-mm tubes with a 12-h photoperiod, and tested for absence of SCYLV by RT-PCR after total RNA purification as described below. SCYLV-free plantlets then were transferred in sterile tubes (10 plantlets per tube) to Guadeloupe and Réunion Island for transmission experiments. Before inoculation with SCYLV, each plantlet was transferred to fresh growth medium in a single 115-by-30-mm polypropylene tube.

Inoculum sources of SCYLV genotypes. Sugarcane stalks infected by the different SCYLV genotypes and healthy control stalks were sampled in the field. Cuttings from these stalks were grown in pots in greenhouses. To avoid cross contamination, plants infected by different virus genotypes were grown in different greenhouses (Réunion Island) or in insect-proof cages in the same greenhouse (Guadeloupe). Sugarcane cvs. B37-161, B86-409, and R570 infected with SCYLV genotypes BRA-PER, CUB, and REU, respectively, were used as inoculum sources in Guadeloupe. Sugarcane cv. "SP71-6163" (mislabeled cultivar) (2) infected by SCYLV genotype BRA-PER or by a mixture of BRA-PER and REU, and cv. R577 infected by genotype REU, were used as inoculum sources in Réunion Island. Genotype identification was performed by RT-PCR with genotype-specific primers as described bellow. Additionally, SCYLV-free sugarcane cvs. B59-92 and R577 were used as healthy control plants in Guadeloupe and Réunion Island, respectively.

Sampling and rearing of *M. sacchari*. The SCYLV aphid vector *M. sacchari* was collected in Guadeloupe and Réunion Island from sugarcane plants that tested negative for SCYLV by tissue-blot immunoassay (TBIA). In order to obtain virus-free aphids, *M. sacchari* individuals were reared on healthy sugarcane leaves of cv. B59-92 in Guadeloupe and cv. R577 in Réunion Island. Rearing was performed with 2- to 4-month-old plants grown in pots and covered with insect-proof cages in Guadeloupe, whereas rearing was performed in glass tubes on detached leaf fragments in Réunion Island (Fig. 1). In Réunion Island, approximately 25-cm-long leaf pieces were taken from the youngest fully emerged leaf of 2- to 4-month-old sugarcane plants grown in a greenhouse, and surface disinfested with an ethanol-soaked cloth under a laminar flow hood. Each leaf fragment was then gently folded, and the two extremities of the U-shaped leaf fragment were inserted in a 200-by-25-mm glass tube containing 20 ml of agar medium (15 g of agar, propiconazole at 25 ppm, and 1 liter of distilled water). Tubes were maintained on the bench in the laboratory at 24ºC for 2 to 3 weeks with a 12-h photoperiod. In both locations, newly born individuals from adult aphids were sampled and reared on new plants or detached leaves, and this procedure was performed for at least two consecutive aphid generations. To produce viruliferous aphids, virus-free stage-four nymphs or adult aphids were reared for 3 to 5 weeks on entire virus-infected plants in a greenhouse in Guadeloupe, and for at least two consecutive generations in the laboratory on detached virus-infected sugarcane leaves in Réunion Island.

Aphid transmission assays. Viruliferous stage-one to -four nymphs of *M. sacchari* were taken from SCYLV-infected leaves and transferred one by one to SCYLV-free tissue-cultured sugarcane plantlets using a paint brush. In each geographical location, 10 to 13 plantlets were inoculated per sugarcane cultivar and per SCYLV genotype, and 7 to 10 nymphs were deposited on the emerging leaves of each plantlet (Fig. 2). The same procedure was used with control plants, but nymphs of *M. sacchari* were taken from SCYLV-free leaves. The nymphs were allowed to feed on the plantlets for a 48- to 72-h inoculation access period (IAP). Plantlets then were sprayed in the polypropylene tube with an insecticide (imidacloprid at 100 ppm). Within 1 week after the transmission assays, inoculated sugarcane plantlets were transferred to Montpellier and to a growth chamber. Plantlets were grown for 1 month in 7-by-7-by-6.5-cm pots containing peat moss and compost (1:1, vol/vol), and then transferred to a quarantine glasshouse in 11-by-11-by-12-cm pots containing the same culture medium. After 1 month of growth in the quarantine glasshouse, plants were transferred to 5-liter pots containing a mixture of peat moss, compost, and volcanic rock (2:2:1, vol/vol/vol) and grown in a regular greenhouse for another 4 months. Plants were observed for symptom development (yellowing of the leaf midrib and yellowing and necrosis of the leaf blade) every 2 weeks, and tested for SCYLV by TBIA and RT-PCR without RNA purification 5 to 6 months after inoculation as described below. The aphid transmission experiment was performed first in Guadeloupe, and the second experiment was performed in Réunion Island 3 weeks later.

Experimental design in the greenhouse in Montpellier. Plants inoculated with SCYLV in Guadeloupe and in Réunion Island were grown separately in two adjacent greenhouses. In each greenhouse, plants of each cultivar–SCYLV genotype combination were divided arbitrarily into two groups and each group was distributed in one of two adjacent blocks.

Detection of SCYLV in sugarcane by TBIA. TBIA with the youngest fully emerged leaf was performed as described by Schenck et al. (30), except that Fast Blue BB salt (Sigma) was used. Membranes were analyzed with a stereomicroscope (×100) to determine positive reactions.

Detection of SCYLV in sugarcane by RT-PCR with RNA purification. Total RNA from the youngest fully emerged leaf was extracted and purified from sugarcane leaves (100 mg of lamina and midrib) with the RNeasy Plant Mini Kit (Qiagen) using the manufacturer's protocol. Total RNA was eluted in a final volume of 40 µl of diethylpyrocarbonate (DEPC)-treated water and stored at –20ºC. RT-PCR was performed using the Ready-To-Go RT-PCR Beads (Amersham Biosciences): 46 µl of DEPC water, 1 µl of each primer YLS111 and YLS462 (10 µM) (1), and 2 µl of eluted RNA were added to each reaction tube. The RT-PCR program was 45ºC for 45 min; 95ºC for 2 min; 35 cycles of 95ºC for 1 min, 60ºC for 1 min, and 72ºC for 1 min; and a final 72ºC extension step for 10 min. The amplified fragment from the coat

Table 1. Sugarcane cultivars used for transmission of different genotypes of *Sugarcane yellow leaf virus* (SCYLV)[v]

Cultivar	Imported from	Resistance level to infection by SCYLV[w]	Resistance level to yellow leaf[x]
CC84-75	Colombia	Highly susceptible (34)	Susceptible[y]
CC85-68	Colombia	Highly susceptible (34)	Unknown[y]
CC85-92	Colombia	Resistant (34)	Unknown[y]
H50-7209	Peru	Unknown	Highly susceptible (3)
H78-3567	Hawaii	Resistant (31)	Resistant (31)
H78-4153	Hawaii	Highly resistant (31)	Resistant (31)
R570	Réunion Island	Unknown	Tolerant (27,28)
SP71-6163	Brazil	Highly susceptible[z]	Highly susceptible (19)

[v] References indicated in parentheses.
[w] Based on experimental data.
[x] Based on leaf yellowing in the field or yield data.
[y] J. Victoria (*personal communication*).
[z] J.-H. Daugrois (*unpublished results*).

protein coding sequence had an expected size of 351 bp. This detection method was applied to all plants that were further used for SCYLV genotype identification.

Detection of SCYLV in sugarcane by RT-PCR without RNA purification. Total RNA was extracted from the midrib of the youngest fully emerged leaf of sugarcane plants. Each midrib fragment (50 mg) was deposited in a 2-ml tube containing two steel pellets (3 mm in diameter). The sample was frozen by immersion of the tube for 30 s in liquid nitrogen, and then homogenized with the Retsch MM301 extractor (Fisher Bioblock Scientific) three times for 30 s each. Carbonate coating buffer (500 µl; 2% [wt/vol] PVP-40 and 1% [wt/vol] Na_2SO_3) was added to each tube, which was centrifuged at 8,800 rpm for 5 min, and the supernatant was stored at 4ºC. For RT-PCR assay, 25 µl of extracted RNA was incubated overnight at 4ºC in 200-µl microcentrifuge tubes. These tubes were washed two times with phosphate-buffered saline (PBS)-Tween 20 and once with DEPC water. RT-PCR was performed using the Titan One Tube RT-PCR System kit (Roche). The 25-µl RT-PCR mix consisted of 5 µl of RT-PCR buffer (5×), 1.25 µl of dithiothreitol solution (100 mM), 0.25 µl of RNase inhibitor (40 U μl^{-1}), 0.5 µl of dNTP mix (10 mM), 0.5 µl of Titan enzyme mix, 0.1 µl of each primer YLS111 and YLS462 (100 µM) (1), and DEPC water to final volume. The RT-PCR program was 54ºC for 60 min; 94ºC for 2 min; 30 cycles of 94ºC for 15 s, 61ºC for 2 min, and 68ºC for 2 min; and a final 68ºC extension step for 10 min. This RT-PCR method was applied to detect SCYLV in all inoculated plants grown in the greenhouse in Montpellier that tested negative by TBIA.

Detection of SCYLV in aphids. Total RNA of three to five pooled aphids was extracted and purified with the RNeasy Plant Mini Kit (Qiagen) using the manufacturer's protocol. Total RNA was eluted in a final volume of 40 µl of DEPC water and stored at –20ºC. RT-PCR with diagnostic primers YLS111 and YLS462 was performed using the Ready-To-Go RT-PCR Beads (Amersham Biosciences) as described above.

SCYLV genotype identification. SCYLV genotypes were identified by RT-PCR using specific primers as described by Abu Ahmad et al. (2). The genotype was determined in each plant used as inoculum source. Two samples of three to five aphids per inoculum source were used to identify the SCYLV genotypes in insects reared on entire plants or detached leaves. At least two infected plants from each sugarcane cultivar–inoculum combination were used in each transmission trial, except when infected plants were not available.

Statistical analyses. The logit $\{\log [p/(1 - p)]\}$ of the infection probability p was analyzed for the sugarcane cultivars and the SCYLV genotypes. The generalized linear model was used (21), and the number x_{ij} of infected plants of sugarcane cultivar i and virus genotype j was distributed as a binomial $B(n_{ij};p_{ij})$. The logit of p_{ij} expectation was written as the linear model $\log \{p_{ij}/[1 - p_{ij}]\} = \mu + \alpha_i + \beta_j$, where α_i was the effect of sugarcane cultivar i and β_j the effect of SCYLV genotype j. The generalized linear model was performed with Genmod procedure of SAS software (release 8.2; SAS Institute Inc., Cary, NC). Quality of adjustment was tested with Pearson's χ^2 test to evaluate the need of an overdispersion parameter (ϕ adjusted on Pearson's χ^2 statistic) in comparison with the binomial distribution. This overdispersion also could be considered as a cultivar–genotype interaction. The sugarcane cultivar and virus genotype effects were tested by the χ^2 test when the model was a good fit. These two effects were tested by the Fisher-Snedecor (F) test when a multiplicative overdispersion was found, and incidences of virus genotypes then were compared for each sugarcane cultivar with Fisher's exact test.

The logit model could not be applied to symptomatic plant data because the number of symptomatic plants was low or nil for several sugarcane cultivars and for SCYLV genotype REU. For this reason, the symptomatic plant data were analyzed

Fig. 1. Rearing of the aphid *Melanaphis sacchari* on detached sugarcane leaves. **A,** Nymphs of *M. sacchari* on a sugarcane leaf fragment. **B,** U-shaped sugarcane leaf fragments in glass tubes.

with Fisher's exact test using the proc Freq procedure of SAS software (release 8.2; SAS Institute Inc.). Cumulated average numbers of symptomatic plants were analyzed separately for the sugarcane cultivars and the SCYLV genotypes. Pairwise comparisons of sugarcane cultivars and of SCYLV genotypes also were performed with Fisher's exact test.

RESULTS

Presence of SCYLV in sugarcane plants and aphids used for transmission of the virus. SCYLV was detected and the virus genotype determined in all plants used for *M. sacchari* rearing (Tables 2 to 5). The virus also was found by RT-PCR in the 12 pooled aphid samples that were taken arbitrarily among the reared insect populations. The genotypes of SCYLV present in these aphids were identical to the virus genotypes present in the plants on which the aphids were reared. The virus was not detected in the healthy control plants and in the aphids reared on these plants.

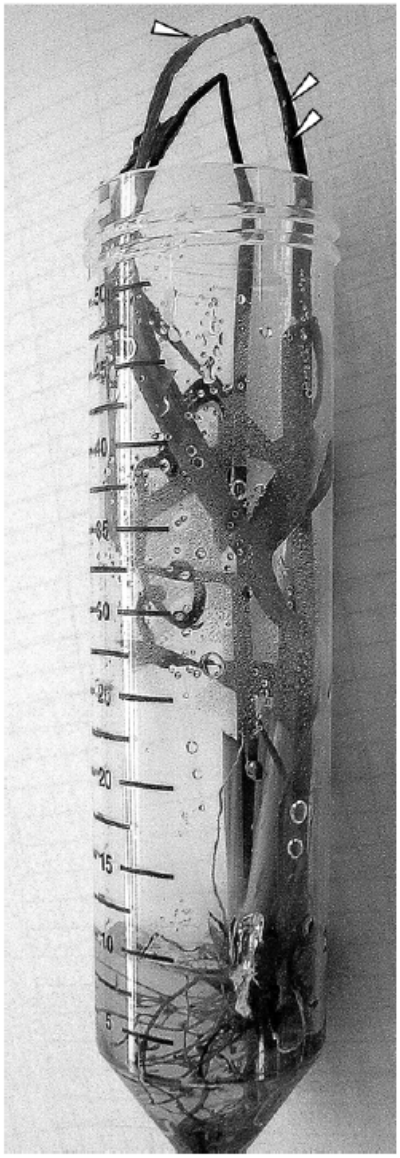

Fig. 2. Tissue-cultured plantlet inoculated with *Sugarcane yellow leaf virus*. Arrows indicate nymphs of *Melanaphis sacchari*.

SCYLV transmission efficiency. In all, 10 to 13 tissue-cultured plantlets were inoculated for each sugarcane cultivar–SCYLV genotype combination in Guadeloupe and in Réunion Island (with the exception of control plants of cvs. CC84-75, CC85-68, CC85-92, and H78-3567 that were not available in Réunion Island). Because some plantlets failed to grow after transfer from the glass tube to the greenhouse, the final number of inoculated plants varied from 2 to 13 (Tables 2 to 5). SCYLV was detected by TBIA in 86 (39%) of 218 plants inoculated in Guadeloupe 5 and 6 months after plant inoculation. Additionally, 20 TBIA-negative plants tested positive by RT-PCR 6 months after inoculation, and 106 (49%) of 218 plants were found infected by the virus. SCYLV was detected by TBIA in 39 (22%) and 41 (23%) of 175 plants inoculated in Réunion Island 5 and 6 months after plant inoculation, respectively. Additionally, 35 TBIA-negative plants tested positive by RT-PCR 6 months after inoculation, and 76 (43%) of 175 plants were found to be infected by the virus.

Infection data 6 months after inoculation were analyzed statistically. No data correction was needed to take into account a sugarcane cultivar–virus genotype interaction for the transmission trial performed in Guadeloupe (overdispersion parameter $\phi = 1.15$, $P = 0.3105$), and the cultivar and genotype effects were significant at $P < 0.0001$ and $P = 0.0077$, respectively (Table 2). In contrast, an overdispersion parameter was needed for the transmission trial performed in Réunion Island ($\phi = 3.33$, $P < 0.0001$), and no significant sugarcane cultivar or SCYLV genotype effect was found ($P = 0.2866$ and 0.3042, respectively). The overdispersion of these latter data compared with a binomial distribution of infected plants was attributed to a sugarcane cultivar–virus genotype interaction in this trial, and this interaction was evident for several cultivars, especially for H78-4153 and R570 (Table 3).

In Guadeloupe, the transmission rate of SCYLV genotype CUB (62% of infected plants) was higher than transmission rates of genotypes BRA-PER (47%) and REU (39%) that were not significantly different (Table 2). In Réunion Island, transmission rates of genotypes BRA-PER (56%) and REU (33%) were not significantly different either; however, this result may have been biased by the overdispersion of data (Table 3). The transmission rate of genotype mixture BRA-PER + REU (42%) was not different from the transmission rates of genotypes BRA-PER and REU transmitted separately.

Variation in SCYLV infection capacity. SCYLV genotype BRA-PER was detected in at least 2 of 10 inoculated plants of all eight sugarcane cultivars in Guadeloupe and Réunion Island. Similarly, genotype CUB was found in at least 3 of 10 inoculated plants of all eight sugarcane cultivars in Guadeloupe. None of the 40 plants of highly resistant sugarcane cvs. H78-3567 and H78-4153 inoculated with genotype REU in Guadeloupe and in Réunion Island was infected by this genotype 6 months after plant inoculation (Tables 2 and 3). In contrast, 13 of 32 plants of these two cultivars were infected by genotype BRA-PER.

SCYLV genotype REU was detected in six of nine plants of sugarcane cv. R570 inoculated with this genotype in Guadeloupe (Table 2), whereas it was not found in any of the nine plants of sugarcane cv. R570 inoculated with genotype REU in Réunion Island (Table 3). Additionally, the five plants of cv. R570 infected by SCYLV after inoculation with the mixture of genotypes BRA-PER + REU all were infected by genotype BRA-PER only. Similarly, only genotype BRA-PER was detected in the SCYLV-positive plant of cv. H78-4153 inoculated with this genotype mixture. Genotypes BRA-PER and REU were detected in the SCYLV-infected plants of all the other sugarcane cultivars.

Variation in SCYLV virulence based on disease symptoms. Symptoms of leaf yellowing were observed first in the greenhouse 3 months after inoculation of plants with SCYLV. At the end of the experiments (6 months after plant inoculation), 31 (14%) of 218 plants inoculated in Guadeloupe and 29 (17%) of 175 plants inoculated in Réunion Island exhibited disease symptoms (Tables 4 and 5). Genotypes BRA-PER, CUB, and REU incited symptoms in 11 of 73 (15%), 17 of 68 (25%), and 3 of 77 (4%) plants inoculated in Guadeloupe, respectively. Genotype BRA-PER, the mixture of genotype BRA-PER and REU, and genotype REU incited symptoms in 14 of 57 (25%), 15 of 55 (27%), and 0 of 63 (0%) plants inoculated in Réunion Island, respectively. Highly significant sugarcane cultivar ($P < 0.0001$) and SCYLV genotype ($P < 0.0015$) effects were found in both experiments, and the percentage of symptomatic plants inoculated with genotype BRA-PER was higher than the percentage of symptomatic plants inoculated with genotype REU (Tables 4 and 5). None of the 103 control plants exhibited yellow leaf symptoms. Four plants of highly susceptible sugarcane cvs. H50-7209 and SP71-6163, inoculated with SCYLV genotype BRA-PER in Guadeloupe, showed disease symptoms (Table 4), although the virus was not detected in these plants (Table 2). Similarly, one plant of susceptible cv. CC84-75 inoculated with genotype BRA-PER, and three plants of highly susceptible cv. H50-7209 inoculated with mixed genotypes BRA-PER and REU in Réunion Island, showed yellow leaf

symptoms (Table 5), although the virus was not detected in these plants (Table 3).

No plant of highly resistant cvs. H78-3567 and H78-4153 and tolerant cv. R570 exhibited disease symptoms, whatever the SCYLV genotype and the geographical location where the plants were inoculated. Genotype BRA-PER from Guadeloupe incited symptoms in highly susceptible sugarcane cvs. H50-7209 and SP71-6163 (Table 4). Genotype BRA-PER from Réunion Island, alone or in mixture with genotype REU, incited symptoms in the same cultivars and in three additional cultivars (Table 5).

Variation in sugarcane resistance to infection by SCYLV. The percentage of plants infected by SCYLV varied between 22% (resistant cv. H78-3567 and highly resistant cv. H78-4153) and 68% (highly susceptible cvs. CC84-75 and SP71-6163) in the transmission experiment performed in Guadeloupe (Table 2), and between 10% (resistant cv. H78-3567) and 71% (highly susceptible cv. CC85-68) in the transmission experiment performed in Réunion Island (Table 3). The percentages of infected plants of resistant cv. H78-3567 and highly resistant cv. H78-4153 were different from the percentages of infected plants of cvs. CC84-75 (highly susceptible), CC85-68 (highly susceptible), CC85-92 (resistant), R570 (unknown resistance to infection), and SP71-6163 (highly susceptible), but not from cv. H50-7209 (resistance to infection unknown but highly susceptible to yellow leaf) in the transmission experiment performed in Guadeloupe (Table 2). No significant differences of infection percentages were found between sugarcane cultivars in the transmission experiment performed in Réunion Island (Table 3); however, this result may have been biased by the overdispersion of data and existence of a sugarcane cultivar–SCYLV genotype interaction in this trial (Table 3). In this experiment, as shown above, plants of sugarcane cvs. H78-3567, H78-4153, and R570 were infected by SCYLV genotype BRA-PER but not by genotype REU.

Variation in sugarcane resistance to yellow leaf. The percentage of plants showing disease symptoms varied between 0% (sugarcane cv. CC85-92, whose resistance to yellow leaf is unknown; resistant sugarcane cvs. H78-3567 and H78-4153; and tolerant cv. R570) and 52% (highly susceptible cv. SP71-6163) in the transmission experiment performed in Guadeloupe (Table 4), and between 0% (resistant sugarcane cvs. H78-3567 and H78-4153 and tolerant cv. R570) and 62% (highly susceptible cv. H50-7209) in the transmission experiment performed in Réunion Island (Table 5).

DISCUSSION

In this study, two different rearing methods and an unusual plant inoculation method were used successfully to transmit SCYLV from diseased to healthy sugarcane plants. Rearing *M. sacchari* aphids on infected sugarcane plants has been described and used for SCYLV transmission by several authors (18,29,31), but this is the first report of rearing *M. sacchari* on detached sugarcane leaves in the laboratory. Additionally, in vitro inoculation of tissue-cultured sugarcane plantlets allowed us to inoculate eight sugarcane cultivars

Table 2. Incidence of three *Sugarcane yellow leaf virus* (SCYLV) genotypes after transmission to eight sugarcane cultivars in Guadeloupe

	SCYLV genotype (sugarcane host cultivar)[x]				
Cultivar (resistance)[y]	Control (B59-92)	BRA-PER (B37-161)	CUB (B86-409)	REU (R570)	Total no. (%)[z]
CC84-75 (HS)	0/8	7/10	6/7	4/8	17/25 (68) a
CC85-68 (HS)	0/9	5/9	7/10	5/10	17/29 (59) a
CC85-92 (R)	0/10	4/7	5/10	4/10	13/27 (48) a
H50-7209 (unknown)	0/10	3/10	5/8	4/8	12/26 (46) a,b
H78-3567 (R)	0/9	2/10	4/9	0/9	6/28 (22) b
H78-4153 (HR)	0/7	4/9	3/10	0/13	7/32 (22) b
R570 (unknown)	0/4	5/9	6/8	6/9	17/26 (65) a
SP71-6163 (HS)	0/11	4/9	6/6	7/10	17/25 (68) a
Total no. (%)[z]	0/68 (0)	34/73 b (47)	42/68 a (62)	30/77 b (39)	…

[x] Number of SCYLV infected plants/total number of inoculated plants; infected plants reacted positively by tissue-blot immunoassay or reverse-transcription polymerase chain reaction 6 months after plant inoculation.
[y] Sugarcane cultivar (resistance level to infection by SCYLV). HS = highly susceptible, R = resistant, and HR = highly resistant.
[z] Values followed by the same letter are not significantly different at $P = 0.05$ according to the χ^2 pairwise test (data of control plants were not included in the statistical analysis).

Table 3. Incidence of two *Sugarcane yellow leaf virus* (SCYLV) genotypes after transmission to eight sugarcane cultivars in Réunion Island

	SCYLV genotype (sugarcane host cultivar)[u]					
Cultivar (resistance)[v]	Control (R577)	BRA-PER ("SP71-6163")[w]	BRA-PER + REU ("SP71-6163")[w]	REU (R577)	Total no. (%)[x]	*P* value[y]
CC84-75 (HS)	…	2/10	…	2/4	4/14 (29)	0.5205
CC85-68 (HS)	…	5/10	10/10	7/11	22/31 (71)	0.0308
CC85-92 (R)	…	7/10	2/10	7/9	16/29 (55)	0.0323
H50-7209 (unknown)	0/8	2/2	3/6	3/5	8/13 (62)	0.7669
H78-3567 (R)	…	2/5	0/7	0/9	2/21 (10)	0.0476
H78-4153 (HR)	0/10	5/8	1[z]/9	0/8	6/25 (24)	0.0115
R570 (unknown)	0/8	3/3	5[z]/8	0/9	8/20 (40)	0.0007
SP71-6163 (HS)	0/9	6/9	2/5	2/8	10/22 (45)	0.3044
Total no. (%)[y]	0/35 (0)	32/57 (56)	23/55 (42)	21/63 (33)	…	…

[u] Number of SCYLV infected plants/total number of inoculated plants; infected plants reacted positively by tissue-blot immunoassay or reverse-transcription polymerase chain reaction 6 months after plant inoculation.
[v] Sugarcane cultivar (resistance level to infection by SCYLV). HS = highly susceptible, R = resistant, and HR = highly resistant.
[w] Mislabeled sugarcane cultivar (2).
[x] Total (%) except control.
[y] No overall significant differences were found between sugarcane cultivars and SCYLV genotypes according to the Fisher Snedecor (F) test at $P = 0.05$ (data of control plants were not included in the statistical analysis). However, significant sugarcane cultivar x virus genotype interaction was found with the over-dispersion test, and virus effect for each cultivar was determined by the *P* value of Fisher's exact test.
[z] Plants infected by SCYLV genotype BRA-PER only; infected plants of all other sugarcane cultivars inoculated with genotype mixture BRA-PER + REU were infected by the two genotypes.

with different SCYLV isolates in two geographical locations (Guadeloupe and Réunion Island), and to study inoculated plants in a single environment (Montpellier) with almost no risk of subsequent cross-contamination between plants. Indeed, *M. sacchari* does not exist in mainland France and the only putative vector of SCYLV existing on maize in the South of France, *R. maidis*, does not colonize sugarcane in our local conditions (G. Labonne, *unpublished results*).

Several differences observed between SCYLV isolates or genotypes suggested variation in infection capacity and virulence of this virus. Percentage of infected and symptomatic plants inoculated with SCYLV genotype CUB was higher than the percentage of infected plants inoculated by genotypes BRA-PER or REU. This difference must, however, be confirmed by additional experiments in Guadeloupe because genotype CUB has not been found in Réunion Island (2). No difference in overall transmission rate was found by statistical analysis between SCYLV genotypes BRA-PER and REU in the experiments performed in Guadeloupe and in Réunion Island. However, variation in infection capacity and virulence based on sugarcane infection and disease symptom development, respectively, was observed between these two genotypes. Whereas sugarcane cvs. H78-3567 and H78-4153 were successfully inoculated with SCYLV genotype BRA-PER in Guadeloupe and in Réunion Island, the virus was never detected in plants of these two cultivars inoculated with genotype REU. This sugarcane cultivar–SCYLV genotype interaction is proof that genotypes BRA-PER and REU are two strains of SCYLV.

Additionally, infection capacity of genotype REU varied according to the geographical origin of the members of this virus group. Genotype REU was transmitted successfully to sugarcane cv. R570 in Guadeloupe, but transmission failed in Réunion Island with two different inoculum sources of the pathogen: genotype REU was not detected in plants of cv. R570 inoculated in Réunion Island with this genotype alone or with a mixture of genotypes BRA-PER and REU. The failure of transmission of SCYLV genotype REU to sugarcane cv. R570 in Réunion Island may explain why incidence of the virus did not significantly vary in a recent 3-year survey of several R570 fields in this geographical location (27). Similar results were obtained recently in Colombia, where an SCYLV isolate was transmitted successfully via *M. sacchari* to several sugarcane cultivars, except to healthy plants of the sugarcane cultivar from which it originated (J. Victoria, *personal communication*).

The failure of transmission of SCYLV genotype REU to sugarcane cv. R570 suggests that this virus genotype evolved in Réunion Island during the last decades because sugarcane cv. R570 was created in 1964 by CERF in Réunion Island and plants of R570 infected by SCYLV genotype REU exist in this geographical location. Further investigations will be necessary to identify the genetic origin of this pathogenic variation in SCYLV genotype REU.

The number of symptomatic plants was not related to plant infection, and 122 of 182 plants infected by SCYLV did not exhibit disease symptoms, thus confirming previous field observations (16,29,33). However, differences in number of symptomatic plants confirmed conclusions based on infection data. Genotype REU caused almost no symptoms in the plants inoculated with this genotype in Guadeloupe and Réunion Island, whereas 15 to 25% of plants inoculated with genotype BRA-PER were symptomatic. Therefore, SCYLV genotype BRA-PER is a more aggressive strain than genotype REU. Pathogenicity variation within genotype BRA-PER also may exist because this genotype from Réunion Island caused symptoms in more cultivars than the same genotype from Guadeloupe. These results, however, must be confirmed by additional or repeated data. Additionally, SCYLV was

Table 4. Number of symptomatic plants of eight sugarcane cultivars inoculated with three *Sugarcane yellow leaf virus* (SCYLV) genotypes in Guadeloupe

	SCYLV genotype (sugarcane host cultivar)[x]				
Cultivar (resistance)[y]	Control (B59-92)	BRA-PER (B37-161)	CUB (B86-409)	REU (R570)	Total no. (%)[z]
CC84-75 (S)	0/8	0/10	4/7	0/8	4/25 (16) b
CC85-68 (unknown)	0/9	0/9	3/10	0/10	3/29 (10) b,c
CC85-92 (unknown)	0/10	0/7	0/10	0/10	0/27 (0) c
H50-7209 (HS)	0/10	5/10	5/8	1/8	11/26 (42) a
H78-3567 (R)	0/9	0/10	0/9	0/9	0/28 (0) c
H78-4153 (R)	0/7	0/9	0/10	0/13	0/32 (0) c
R570 (T)	0/4	0/9	0/8	0/9	0/26 (0) b,c
SP71-6163 (HS)	0/11	6/9	5/6	2/10	13/25 (52) a
Total no. (%)[z]	0/68 (0)	11/73 a (15)	17/68 a (25)	3/77 b (4)	…

[x] Number of plants with yellow leaf symptoms/total number of inoculated plants.
[y] Sugarcane cultivar (resistance level to yellow leaf). HS = highly susceptible, S = susceptible, T = tolerant, and R = resistant.
[z] Values followed by the same letter are not significantly different at $P = 0.05$ according to Fisher's exact test (data of control plants were not included in the statistical analysis).

Table 5. Number of symptomatic plants of eight sugarcane cultivars inoculated with two *Sugarcane yellow leaf virus* genotypes in Réunion Island

	SCYLV genotype (sugarcane host cultivar)[v]				
Cultivar (resistance)[w]	Control (R577)	BRA-PER ("SP71-6163")[x]	BRA-PER + REU ("SP71-6163")[x]	REU (R577)	Total no. (%)[y]
CC84-75 (S)	…	3/10	…	0/4	3/14 (21) a,b,c
CC85-68 (unknown)	…	4/10	6/10	0/11	10/31 (32) a
CC85-92 (unknown)	…	1/10	1/10	0/9	2/29 (7) b,c
H50-7209 (HS)	0/8	2/2	6/6	0/5	8/13 (62) a
H78-3567 (R)	…	0/5	0/7	0/9	0/21 (0) c
H78-4153 (R)	0/10	0/8	0/9[z]	0/8	0/25 (0) c
R570 (T)	0/8	0/3	0/8[z]	0/9	0/20 (0) c
SP71-6163 (HS)	0/9	4/9	2/5	0/8	6/22 (27) a,b
Total no. (%)[y]	0/35 (0)	14/57 a (25)	15/55 a (27)	0/63 b (0)	…

[v] Number of plants with yellow leaf symptoms/total number of inoculated plants.
[w] Sugarcane cultivar (resistance level to yellow leaf). HS = highly susceptible, S = susceptible, T = tolerant, and R = resistant.
[x] Mislabeled sugarcane cultivar (2).
[y] Values followed by the same letter are not significantly different at $P = 0.05$ according to Fisher's exact test (data of control plants were not included in the statistical analysis).
[z] Plants infected by SCYLV genotype BRA-PER only.

not detected in several plants showing yellow leaf symptoms but inoculated with SCYLV genotype BRA-PER. Therefore, these symptoms were caused by virus populations of this genotype not detected by TBIA and RT-PCR, or by an undetermined physiological disorder.

In Hawaii, sugarcane cv. H78-4153 is highly resistant to infection by SCYLV (31). In this geographical location, cv. H78-4153 remained virus-free in SCYLV-contaminated fields over a 1-year period of observation, and all aphid-inoculated plants tested negative for SCYLV (31). In contrast, in this study, H78-4153 was infected by genotype BRA-PER in Réunion Island and in Guadeloupe, and also by genotype CUB in Guadeloupe. These differences in transmission efficiency may be additional evidence for variation in infection capacity of SCYLV or may be caused by variation in biotypes of *M. sacchari* in the different geographical locations. Virus transmission is correlated with host and environment adaptation in several aphid species (4,13,14,26). Additionally, several field populations or clones of *M. sacchari* differing in their ability to transmit SCYLV may exist, as was shown for the aphids *R. padi* and *Sitobion avenae* and transmission of different *Barley yellow dwarf virus* serotypes (9,20).

This study is the first report of a rearing method of *M. sacchari* on detached sugarcane leaves, efficient inoculation of tissue-cultured sugarcane plantlets with SCYLV, and variation in infection capacity and virulence of SCYLV. Additional studies are needed to further investigate the importance of SCYLV strains on a world-scale basis and to identify the genetic origin of this variation. Segregation for resistance exists in sugarcane progeny and resistance to infection by SCYLV appears to be a promising method to control yellow leaf (34). However, efficient screening of resistant sugarcane cultivars will have to take into account variation in infection capacity and virulence of SCYLV.

ACKNOWLEDGMENTS

This research was conducted during the thesis scholarship program of Youssef Abu Ahmad, supported by the Government of Syrian Arabic Republic and CENICAÑA in Colombia. We thank M.-J. Darroussat, R. Boisne-Noc, M. Payet, J.-F. Bousquet, E. Fernandez, R. Habas, and M. Muller for technical assistance; G. Labonne, J.-M. Lett, B. Lockhart, and N. Sauvion for fruitful discussions and suggestions regarding aphid rearing; B. Vercambre for *Melanaphis sacchari* aphid identification; AIPSA (Peru), CENICAÑA (Colombia), CERF (Réunion Island), COPERSUCAR (Brazil), and HARC (Hawaii) for supplying sugarcane cultivars used in this study; and S. Schenck, E. Mirkov, and J. Victoria for critical review of the manuscript.

LITERATURE CITED

1. Abu Ahmad, Y., Rassaby, L., Royer, M., Borg, Z., Braithwaite, K. S., Mirkov, T. E., Irey, M. S., Perrier, X., Smith, G. R., and Rott, P. 2006. Yellow leaf of sugarcane is caused by at least three different genotypes of sugarcane yellow leaf virus, one of which predominates on the Island of Réunion. Arch. Virol. 151:1355-1371.
2. Abu Ahmad, Y., Royer, M., Daugrois, J.-H., Costet, L., Lett, J.-M., Victoria, J. I., Girard, J.-C., and Rott, P. 2006. Geographical distribution of four *Sugarcane yellow leaf virus* genotypes. Plant Dis. 90:1156-1160.
3. Alegria, O. M., Chatenet, M., Girard, J.-C., Saldarriaga, S. A., Nuget, A., and Rott, P. 2000. First report of *Sugarcane yellow leaf virus* in Peru. Plant Dis. 84:1342.
4. Anstead, J. A., Burd, J. D., and Shufran, K. A. 2002. Mitochondrial DNA sequence divergence among *Schizaphis graminum* (Hemiptera: Aphididae) clones from cultivated and non-cultivated hosts: haplotype and host associations. Bull. Entomol. Res. 92:17-24.
5. Borg, Z., Moonan, F., Braithwaite, K., Mirkov, T. E., and Smith, G. 2001. Characterising the genetic diversity of sugarcane yellow leaf virus. Proc. Int. Soc. Sugar Cane Technol. Congr. 24:654-656.
6. Chatenet, M., Delage, C., Ripolles, M., Irey, M., Lockhart, B. E. L., and Rott, P. 2001. Detection of *Sugarcane yellow leaf virus* in quarantine and production of virus-free sugarcane by apical meristem culture. Plant Dis. 85:1177-1180.
7. Comstock, J. C., Irey, M. S., Lockhart, B. E. L., and Wang, Z. K. 1998. Incidence of yellow leaf syndrome in CP cultivars based on polymerase chain reaction and serological techniques. Sugar Cane 4:21-24.
8. D'Arcy, C. J., and Domier, L. L. 2005. *Luteoviridae*. Pages 891-900 in: Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses. C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball, eds. Elsevier Academic Press, London.
9. Dedryver C.-A., Riault, G., Tanguy, S., Le Gallic, J. F., Trottet, M., and Jacquot, E. 2005. Intra-specific variation and inheritance of BYDV-PAV transmission in the aphid *Sitobion avenae*. Eur. J. Plant Pathol. 111:341-354.
10. Fontaniella, B., Vicente, C., Legaz, M. E., de Armas, R., Rodríguez, C. W., Martinez, M., Piñón, D., Acevedo, R., and Solas, M. T. 2003. Yellow leaf syndrome modifies the composition of sugarcane juices in polysaccharides, phenols and polyamines. Plant Physiol. Biochem. 41:1027-1036.
11. Gonçalves, M. C., Vega, J., Olivieira, J. G., and Gomes, M. A. 2005. Sugarcane yellow leaf virus infection leads to alterations in photosynthetic efficiency and carbohydrate accumulation in sugarcane leaves. Fitopatol. Bras. 30:10-16.
12. Gray, S. M., and Banerjee, N. 1999. Mechanisms of arthropod transmission of plant and animal viruses. Microbiol. Mol. Biol. Rev. 63:128-148.
13. Gray, S., and Gildow, F. E. 2003. Luteovirus–aphid interactions. Annu. Rev. Phytopathol. 41:539-566.
14. Gray, S. M., Smith, D. M., Barbierri, L., and Burd, J. 2002. Virus transmission phenotype is correlated with host adaptation among genetically diverse populations of the aphid *Schizaphis graminum*. Phytopathology 92:970-975.
15. Grisham, M. P., Pan, Y.-B., Legendre, B. L., Godshall, M. A., and Eggleston, G. 2001. Effect of sugarcane yellow leaf virus on sugarcane yield and juice quality. Proc. Int. Soc. Sugar Cane Technol. Congr. 24:434-438.
16. Grisham, M. P., Pan, Y. B., White, W. H., Godshall, M. A., Legendre, B. L., and Comstock, J. C. 2002. Potential effect of yellow leaf syndrome on the Louisiana sugarcane industry. J. Am. Soc. Sugar Cane Technol. 22:125-126.
17. Izaguirre-Mayoral, M. L., Carballo, O., Alceste, C., Romano, M., and Nass, H. A. 2002. Physiological performance of asymptomatic and yellow leaf syndrome-affected sugarcanes in Venezuela. J. Phytopathol. 150:13-19.
18. Lehrer, A. T., Schenck, S., Fitch, M. M. M., Moore, P. H., and Komor, E. 2001. Distribution and transmission of sugarcane yellow leaf virus (SCYLV) in Hawaii and its elimination from seedcane. Proc. Int. Soc. Sugar Cane Technol. Congr. 24:439-443.
19. Lockhart, B. E. L., and Cronjé, C. P. R. 2000. Yellow leaf syndrome. Pages 291-295 in: A Guide to Sugarcane Diseases. P. Rott, R. A. Bailey, J. C. Comstock, B. J. Croft, and A. S. Saumtally, eds. La Librairie du Cirad, Montpellier, France.
20. Lucio-Zavaleta, E., Smith, D. M., and Gray, S. M. 2001. Variation in transmission efficiency among *Barley yellow dwarf virus*-RMV isolates and clones of the normally inefficient aphid vector, *Rhopalosiphum padi*. Phytopathology 91:792-796.
21. McCullagh, P., and Nelder, J. A. 1989. Generalized Linear Models. Second edition. Chapman and Hall, London.
22. Miller, W. A., and Rosochová, L. 1997. Barley yellow dwarf viruses. Annu. Rev. Phytopathol. 35:167-190.
23. Moonan, F., and Mirkov, T. E. 2002. Analyses of genotypic diversity among North, South, and Central American isolates of *Sugarcane yellow leaf virus*: evidence for Colombian origins and for intraspecific spatial phylogenetic variation. J. Virol. 76:1339-1348.
24. Moonan, F., Molina, J., and Mirkov, T. E. 2000. *Sugarcane yellow leaf virus*: an emerging virus that has evolved by recombination between luteoviral and poleroviral ancestors. Virology 269:156-171.
25. Moutia, J.-F. Y., and Saumtally, S. 2001. Diagnosis of sugarcane yellow leaf virus in cane juice and the effect of hot water treatment on its control. Proc. Int. Soc. Sugar Cane Technol. Congr. 24:444-450.
26. Porter, D. R., Burd, J. D., Shufran, K. A., Webster, J. A., and Teetes, G. L. 1997. Greenbug (Homoptera: Aphididae) biotypes: selected by resistant cultivars or preadapted opportunists? J. Econ. Entomol. 90:1055-1065.
27. Rassaby, L., Girard, J.-C., Lemaire, O., Costet, L., Irey, M. S., Kodja, H., Lockhart, B. E. L., and Rott, P. 2004. Spread of *Sugarcane yellow leaf virus* in sugarcane plants and fields on the Island of Réunion. Plant Pathol. 53:117-125.
28. Rassaby, L., Girard, J.-C., Letourmy, P., Chaume, J., Irey, M. S., Lockhart, B. E. L., Kodja, H., and Rott, P. 2003. Impact of *Sugarcane yellow leaf virus* on sugarcane yield and juice quality in Réunion Island. Eur. J. Plant Pathol. 109:459-466.
29. Scagliusi, S. M., and Lockhart, B. E. L. 2000. Transmission, characterization, and serology of a luteovirus associated with yellow leaf syndrome of sugarcane. Phytopathology 90:120-124.
30. Schenck, S., Hu, J. S., and Lockhart, B. E. L. 1997. Use of a tissue blot immunoassay to determine the distribution of sugarcane yellow leaf virus in Hawaii. Sugar Cane 4:5-8.
31. Schenck, S., and Lehrer, A. T. 2000. Factors affecting the transmission and spread of *Sugarcane yellow leaf virus*. Plant Dis. 84:1085-1088.
32. Smith, G. R., Borg, Z., Lockhart, B. E. L., Braithwaite, K. S., and Gibbs, M. J. 2000. *Sugarcane yellow leaf virus*: a novel member of the *Luteoviridae* that probably arose by inter-species recombination. J. Gen. Virol. 81:1865-1869.
33. Vega, J., Scagliusi, S. M. M., and Ulian, E. C. 1997. Sugarcane yellow leaf disease in Brazil: evidence of association with a luteovirus. Plant Dis. 81:21-26.
34. Victoria, J. I., Avellaneda, M. C., Angel, J. C., and Guzmán, M. L. 2005. Resistance to *Sugarcane yellow leaf virus* in Colombia. Proc. Int. Soc. Sugar Cane Technol. Congr. 25:664-670.
35. Viswanathan, R. 2002. Sugarcane yellow leaf syndrome in India: Incidence and effect on yield parameters. Sugar Cane Int. 20:17-23.